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Fatty Acid Metabolism Mediated by 12/15-Lipoxygenase is a Novel Regulator of Hematopoietic Stem Cell Function and Myelopoiesis

Michelle Kinder

University of Pennsylvania, mikinder@gmail.com

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Fatty Acid Metabolism Mediated by 12/15-Lipoxygenase is a Novel Regulator of Hematopoietic Stem Cell Function and Myelopoiesis

Abstract

Fatty acid metabolism governs critical cellular processes in multiple cell types. The goal of my dissertation was to investigate the intersection between fatty acid metabolism and hematopoiesis. Although fatty acid metabolism has been extensively studied in mature hematopoietic subsets during inflammation, in developing hematopoietic cells the role of fatty acid metabolism, in particular by 12/15-Lipoxygenase (12/15-LOX), was unknown. The observation that 12/15-LOX-deficient (Alox15) mice developed a myeloid leukemia instigated my studies since leukemias are often a consequence of dysregulated hematopoiesis. This observation led to the central hypothesis of this dissertation which is that polyunsaturated fatty acid metabolism mediated by 12/15-LOX participates in hematopoietic development. Using genetic mouse models and in vitro and in vivo cell development assays, I found that 12/15-LOX indeed regulates multiple stages of hematopoiesis including the function of hematopoietic stem cells (HSC) and the differentiation of B cells, T cells, basophils, granulocytes and monocytes. Within hematopoietic development, I concentrated on the mechanisms that underlie the defects in HSC function and monocyte development since these defects likely contribute to the myeloid leukemogenesis in Alox15 mice. Interestingly, I determined that 12/15-LOX promotes HSC self-renewal and quiescence, which is associated with the activation of canonical Wnt signaling. Moreover, my studies demonstrate that 12/15-LOX-mediated redox signaling of SHP-2 and the transcription factor ICSBP/IRF-8 promotes monocyte development while inhibiting granulocyte development. This pathway is also conserved in IL-12p40 expression in macrophages. Therefore, I establish 12/15-LOX as a critical regulator of hematopoiesis and provide insight into novel mechanisms whereby HSC function and monocyte cell fate decisions are regulated. These findings have implications for leukemogenesis and immunity.

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**FATTY ACID METABOLISM MEDIATED BY 12/15-LIPOXYGENASE IS A
NOVEL REGULATOR OF HEMATOPOIETIC STEM CELL FUNCTION AND
MYELOPOIESIS**

Michelle Kinder

A Dissertation in
Immunology

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements For the Degree of

Doctor of Philosophy

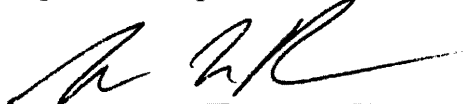
2010

Dissertation Committee

Yvonne Paterson, Ph.D. Professor, Department of Microbiology (Chair)
Michael Atchison, Ph.D. Professor, Department of Animal Biology
Martin P. Carroll, M.D., Associate Professor, Department of Medicine
Warren Pear, M.D. Ph.D. Associate Professor, Department of Pathology and
Laboratory Medicine



Dissertation Advisor: Ellen Puré, Ph.D. Professor, Molecular and Cellular
Oncogenesis Program, Wistar Institute



Graduate Group Chair: Steven Reiner, M.D. Professor Immunology Department,
University of Pennsylvania

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Hematopoietic Stem Cell Function and Myelopoiesis

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Michelle Kinder

DEDICATION

This work is dedicated to my grandparents William and Santina Kinder. My promises to them have driven me towards my career goals. With this dissertation, I am keeping my promises.

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I would like to acknowledge my parents, William and Frances Kinder, who instilled in me the value of hard work, determination, pride and dedication.

Without these values, I would have given up long ago.

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ABBREVIATIONS

5-FU: 5-fluorouracil

AA: Arachidonic Acid

AP-1: activator protein-1

ATM: ataxia telangiectasia mutated

B6: C57BL/6

Blimp-1: B-lymphocyte-induced maturation protein-1

BM: Bone Marrow

BSO: buthionine sulfoximine

BrdU: bromodeoxyuridine

C/EBP α : CCAAT/enhancer binding protein- α

CFU-G: colony-forming units-granulocyte

CFU-GM: colony-forming units- granulocyte macrophage

CFU-M: colony-forming units-macrophage

CLP: common lymphoid progenitor

CML: chronic myelogenous leukemia

CMP: common myeloid progenitor

COX: cyclooxygenase

Dab2: disabled-2

DC: dendritic cells

DP: double positive

EET: epoxyeicosatrienoic acids

Egr-1: early growth response factor-1

Epo: erythropoietin

Erk: extracellular signal-regulated kinase

FCS: fetal calf serum

FoxO: forkhead box subgroup O

FoxP3: forkhead box P3⁺

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

G-CSF: granulocyte colony stimulating factor

Gfi-1: growth factor independent-1

GM-CSF: granulocyte-macrophage stimulating factor

GMP: granulocyte-macrophage progenitors

H&E: hematoxylin and eosin

HCT: hematocrit

HETE: hydroxyeicosatetraenoic acids

HGB: hemoglobin

HODE: hydroxyoctadecadienoic acid

Hox: homeobox

HpETE: hydroperoxyeicosatetraenoic acids

HpODE: hydroperoxyoctadecadienoic acid

HSC: Hematopoietic Stem Cells

ICSBP: interferon consensus sequence binding protein

IFN- γ : interferon- γ

Ig: immunoglobulin

IKK: I κ B kinase

IL-12p40: interleukin-12p40

IRF: interferon regulatory factor

Keap1: kelch like-ECH-associated protein 1

LT: leukotriene

LOX: lipoxygenase

LPS: lipopolysaccharide

LSK: Lin⁻Sca1⁺cKit⁺

LT-HSC: long-term hematopoietic stem cells

LX: lipoxin

M-CSF: macrophage colony stimulating factor

MAPK: mitogen activated protein kinase

MCV: mean cell volumes

MEP: megakaryocyte-erythroid progenitors

mTOR: mammalian target of rapamycin

MPD: myeloproliferative disease

MP: myeloid progenitors

MPN: myeloproliferative neoplasms

MPP: multipotent progenitors

Nab-2: NGFI-A binding protein 2

NAC: N-acetylcysteine

NF-1: neurofibromin

NK: natural killer

NADPH: nicotinamide adenine dinucleotide phosphate

Nrf2: nuclear factor erythroid-derived-like 2

NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells

PBS: phosphate buffered saline

PGE₂: prostaglandin E2

phox91: phagocyte oxidase 91

phosphoinositide 3-kinase PI3-K

PKC: protein kinase C

PLC γ 2: phospholipase C γ 2

PP1 α : protein phosphatase-1 α

PTEN: phosphatase tensin homolog

Rac1: ras-related C3 botulinum toxin substrate 1

RBC: red blood cell

ROS: reactive oxygen species

SCF: stem cell factor

SHP-2: Src homology 2-containing tyrosine phosphatase-2

SLAM: signaling lymphocytic activation molecule

STAT: signal transducers and activators of transcription

ST-HSC: short-term HSCs

TSC: tuberous sclerosis complex

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

WBC: white blood cell

ABSTRACT

FATTY ACID METABOLISM MEDIATED BY 12/15-LIPOXYGENASE IS A NOVEL REGULATOR OF HEMATOPOIETIC STEM CELL FUNCTION AND MYELOPOIESIS

Michelle Kinder

Advisor: Ellen Puré

Fatty acid metabolism governs critical cellular processes in multiple cell types. The goal of my dissertation was to investigate the intersection between fatty acid metabolism and hematopoiesis. Although fatty acid metabolism has been extensively studied in mature hematopoietic subsets during inflammation, in developing hematopoietic cells the role of fatty acid metabolism, in particular by 12/15-Lipoxygenase (12/15-LOX), was unknown. The observation that 12/15-LOX-deficient (Alox15) mice developed a myeloid leukemia instigated my studies since leukemias are often a consequence of dysregulated hematopoiesis. This observation lead to the central hypothesis of this dissertation which is that polyunsaturated fatty acid metabolism mediated by 12/15-LOX participates in hematopoietic development. Using genetic mouse models and *in vitro* and *in vivo* cell development assays, I found that 12/15-LOX indeed regulates multiple stages of hematopoiesis including the function of hematopoietic stem cells (HSC) and the differentiation of B cells, T cells, basophils, granulocytes and monocytes. Within hematopoietic development, I concentrated on the mechanisms that underlie the defects in HSC function and monocyte development since these

defects likely contribute to the myeloid leukemogenesis in Alox15 mice. Interestingly, I determined that 12/15-LOX promotes HSC self-renewal and quiescence, which is associated with the activation of canonical Wnt signaling. Moreover, my studies demonstrate that 12/15-LOX-mediated redox signaling of SHP-2 and the transcription factor ICSBP/IRF-8 promotes monocyte development while inhibiting granulocyte development. This pathway is also conserved in IL-12p40 expression in macrophages. Therefore, I establish 12/15-LOX as a critical regulator of hematopoiesis and provide insight into novel mechanisms whereby HSC function and monocyte cell fate decisions are regulated. These findings have implications for leukemogenesis and immunity.

INTRODUCTION

Hematopoiesis is the dynamic process whereby all blood cells develop from a common progenitor, the hematopoietic stem cell (HSC). Under homeostatic conditions, hematopoiesis must continuously replenish billions of cells of the blood system daily including erythrocytes and neutrophils, which exhibit short half-lives. As HSC are relatively rare, but there is an abundance of blood cells, the process of hematopoiesis must be tightly regulated to produce appropriate numbers of each blood cell type. Moreover, the process of hematopoiesis must adapt to stresses such as infection. Dysregulation of hematopoiesis can result in immunodeficiencies, anemias or leukemias. Therefore, it is important to study the molecular processes that underlie normal and malignant hematopoietic development. In this dissertation, I define polyunsaturated fatty acid metabolism mediated by 12/15-Lipoxygenase (12/15-LOX) as a critical regulator of hematopoiesis. Specifically, I elucidate the mechanisms whereby 12/15-LOX regulates HSC function and myelopoiesis.

Hematopoietic Stem Cells

HSCs are multipotent cells that give rise to all of the cells of the blood during hematopoiesis (Figure 1). HSCs possess the unique ability to undergo asymmetric division by proliferating in a fashion that preserves self-renewal and also allows for differentiation to give rise to mature blood cells. Because markers for HSCs have been identified (Wilson et al., 2007) and assays for measuring HSC function are well defined (Purton and Scadden, 2007),

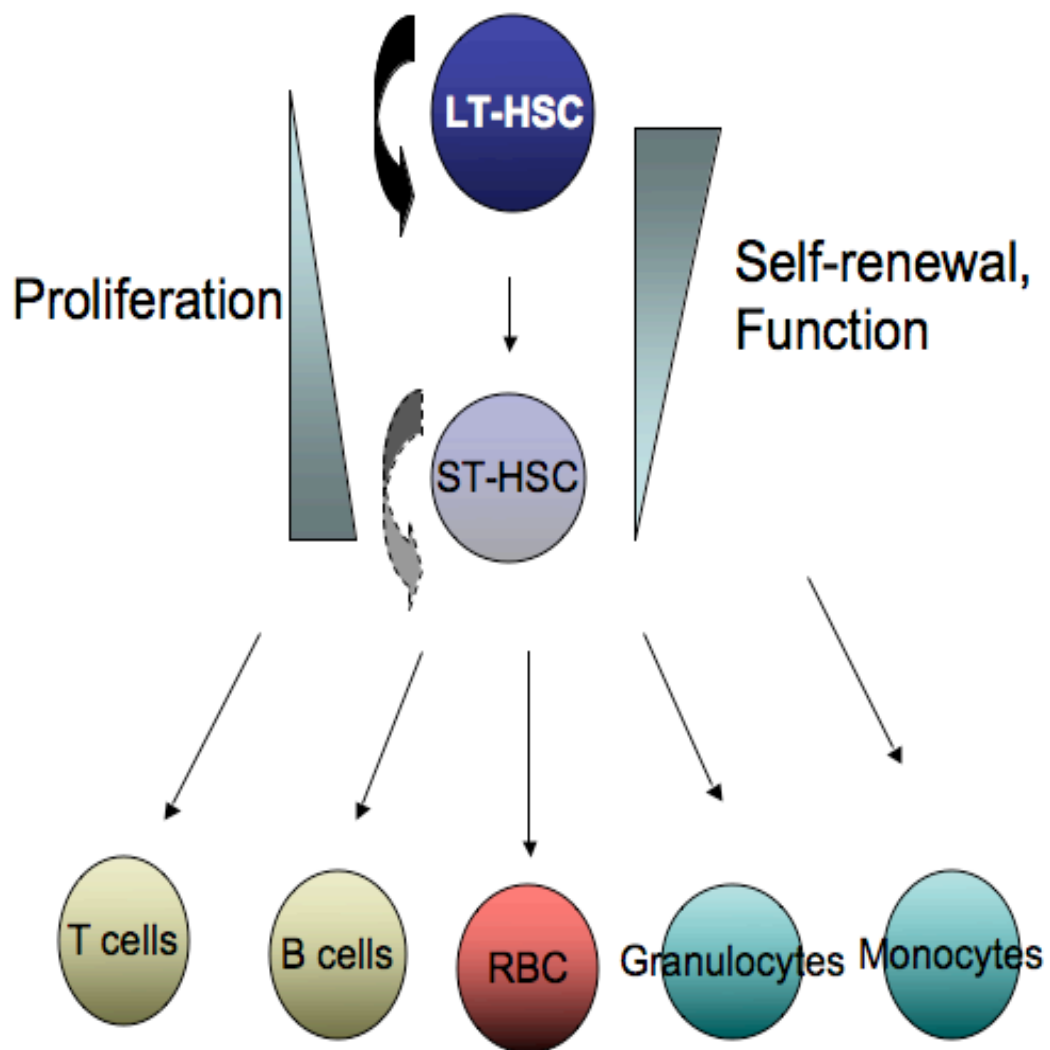


Figure 1. Overview of hematopoiesis. Hematopoiesis is the process whereby the HSC differentiate to all the cells of the blood including lymphoid, erythroid and myeloid lineages. Only HSCs have the ability to self-renew and are enriched within the LSK subset. LT-HSCs are generally quiescent, and have a high capacity to self-renew and function, or reconstitute a lethally irradiated mouse, while ST-HSCs are more proliferative, and have a decreased ability to self-renew and function.

HSCs are an attractive model system for understanding tissue stem cells in general. The study of HSCs provides insight into the regulation of stem cells during infection, aging, and oncogenesis.

HSCs have been identified using various phenotypic markers. Although murine HSCs are enriched within the Lin-Sca1+cKit⁺ (LSK) cell compartment, they can be further subdivided both functionally and phenotypically into multiple subtypes by a combination of other markers including CD34 (Morel et al., 1996), Flt3 (Yang et al., 2005) and the signaling lymphocyte activation molecule (SLAM) markers CD48 and CD150 (Kiel et al., 2005). Long-term HSCs (LT-HSC) are the most primitive HSC and fully reconstitute the hematopoietic compartment of lethally irradiated animals. LT-HSCs are highly enriched (1 of every 2 cells) within the subset of cells that are identified based on phenotypic profile LSKFlt3⁻CD48⁻CD150⁺CD34⁻ (Wilson et al., 2007). They reside in a hypoxic niche and are generally quiescent (Cheshier et al., 1999; Wilson et al., 2007). Recent studies have subdivided the LT-HSC compartment into dormant and activated LT-HSC based on cell retention assays using bromodeoxyuridine (BrdU) and histone-2B-GFP labeling. Dormant LT-HSCs rarely divide but can become activated to undergo asymmetric division in order to respond to environmental stresses such as chemotherapy treatment (for example, 5-fluorouracil, 5-FU), cytokine stimulation and hematopoietic reconstitution. When stimulation wanes and homeostasis is reestablished, activated LT-HSCs become dormant again (Wilson et al., 2008). In comparison, short-term HSCs (ST-HSC) have decreased ability for long term reconstitution of lethally irradiated animals and are more

proliferative than LT-HSCs (Figure 1). ST-HSCs can further differentiate to become multipotent progenitors (MPP), which retain the ability to generate all blood lineages, but lose the potential to self-renew. Subpopulations of MPP may differ in their ability to differentiate into various blood cell types (Wilson et al., 2007).

One way of measuring HSC function is by measuring their ability to reconstitute lethally irradiated hosts. There are multiple factors that contribute to HSC function in this regard (Figure 2). The numbers, homing and survival of HSCs can effect their ability to function. Defective asymmetric division also effects HSC function. Inability to self-renew or differentiate compromises HSC function. The ability of HSCs to self-renew and differentiate is regulated through their environmental niche, cytokine signaling and the orchestrated activities of various transcription factors including growth factor independent-1 (Gfi-1), Bmi, homeobox B4 (Hox4b) and Pu.1 (Blank et al., 2008). However, less is known about the signaling pathways that regulate HSC function. In this dissertation, I demonstrate that signaling downstream of 12/15-LOX-mediated fatty acid metabolism regulates HSC self-renewal.

The unique capacity of HSCs to both differentiate and self-renew is closely related to their proliferative capacity (Ficara et al., 2008; Nemeth et al., 2006). Highly proliferative HSCs are less efficient at reconstituting lethally irradiated mice (Passegue et al., 2005). Thus, HSCs that lack cell cycle inhibitors required to maintain quiescence, such as p21^{cip}/WAF, are functionally defective

Contributing factors in HSC function

- Number of cells
- Localization and signaling within specialized niche
- Survival of HSC
- Proliferation
- Defective Asymmetric Division

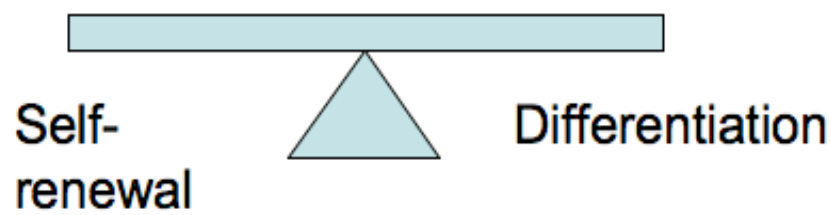


Figure 2. Factors in HSC function. Multiple factors regulate HSC function including the numbers of HSCs, localization and signaling within the specialized stem cell niche, the survival of HSCs, proliferation of HSCs and the ability of HSCs to undergo asymmetric division. Asymmetric division is the balance between self-renewal and differentiation.

(Cheng et al., 2000). This dissertation will demonstrate that HSCs from 12/15-LOX-deficient mice, Alox15, exhibit defective quiescence and HSC function.

Canonical Wnt Signaling

As mentioned, little was known about signaling pathways that regulate HSC function. Canonical Wnt signaling is one such pathway that has been implicated to regulate HSC function although its importance remains debated. Canonical Wnt signaling is believed to regulate HSC self-renewal and is known to play a role in other cell types, such as promoting B and T lymphocyte development (Staal and Sen, 2008) and enhancing self-renewal of tumor cells (Fodde and Brabletz, 2007). There are 19 defined Wnt ligands that are lipid modified by palmitoylation, which is required for its secretion from the cell (Takada et al., 2006; Willert et al., 2003). Wnt ligands act on 10 different Frizzled receptors and two low-density lipoprotein receptor-related protein (LRP) co-receptors.

Wnt signaling is based on the availability of the ligands, receptors and secondary messengers. Up to 10 signaling pathways have been implicated downstream of Wnt signaling. The canonical signaling pathway results in stabilization and nuclear translocation of the transcription factor β -catenin (Malhotra and Kincade, 2009). During canonical signaling, ligands such as Wnt3a or Wnt 10b activate the frizzled receptor and LRP co-receptor to activate disheveled. Disheveled functions by inhibiting the β -catenin destruction complex which is composed of axin, glycogen synthase kinase 3 (GSK-3 β) and adenomatous polyposis coli (APC). In the absence of canonical Wnt

signaling, this complex binds and phosphorylates β -catenin targeting it for ubiquitination and subsequent proteolysis. When members of this complex such as axin are over-expressed, β -catenin protein is degraded and signaling is reduced, resulting in decreased HSC function and self-renewal (Reya et al., 2003). However, in the presence of canonical Wnt signaling β -catenin and a related protein, γ -catenin, are stabilized and can translocate to the nucleus where it acts as a co-activator for the lymphoid enhancer factor (LEF) and T cell factor (TCF) transcription factors (Malhotra and Kincade, 2009). The non-canonical Wnt signaling pathway is activated in part by the Wnt5a ligand and can inhibit canonical Wnt signaling (Nemeth et al., 2007).

The role of canonical Wnt signaling in HSC function has been controversial because inducible deletion of β -catenin and inducible co-deletion of β -catenin/ γ -catenin using Mx-Cre had little effect on HSC function (Cobas et al., 2004; Jeannet et al., 2008; Koch et al., 2008). However, in these studies, the LEF/TCF reporter was still active (Jeannet et al., 2008). On the other hand, recent evidence points to a critical role for canonical Wnt signaling in regulation of HSC quiescence and self-renewal. For instance, deletion of β -catenin using Vav-cre, resulted in defective self-renewal of LT-HSCs (Zhao et al., 2007). HSC function was also compromised due to defective self-renewal when the canonical ligand Wnt3a was deleted (Luis et al., 2009). Over-expression of Dickkopf-1, an inhibitor of canonical Wnt signaling, increased HSC proliferation and decreased HSC self-renewal (Fleming et al., 2008). Conversely, when Wnt signaling was elevated, such as in Hmgb3-deficient HSCs, the ability of HSCs to self-renew

and repopulate following hematopoietic stress were enhanced (Nemeth et al., 2006). Taken together, these data support a role of canonical Wnt signaling in HSC self-renewal. This dissertation defines 12/15-LOX as a novel regulator of canonical Wnt signaling.

Myelopoiesis

Myelopoiesis is the process whereby myeloid cells, monocytes and granulocytes, are generated. Although originally thought of as a linear pathway, recent studies have shown that immature intermediates once thought to give rise exclusively to cells of the lymphoid lineage also possess myeloid cell potential (Bell and Bhandoola, 2008). These studies have caused the field to rethink the hierarchical model of myeloid cell differentiation (Ceredig et al., 2009). However, in order to concentrate on myeloid lineage differentiation rather than cells arising during lymphoid differentiation, I will refer to the classical model of myeloid cell differentiation (Figure 3). However, it is important to note that the lineage decisions between monocytes and granulocytes remain a mutually exclusive cell fate decision that is conserved throughout various models of hematopoiesis.

HSCs are multipotent and can differentiate into any hematopoietic lineage. However, as HSC differentiate they lose their ability to self-renew and are more restricted in their differentiation potential. HSC differentiate into MPP, which lack the ability to self-renew but can still differentiate into all lineages. MPP initiate T cell differentiation by seeding the thymus (Schwarz and Bhandoola, 2004), initiate B cell differentiation by differentiating into common lymphoid progenitors (CLP) (Kondo et al., 1997) or initiate myeloid cell differentiation by becoming

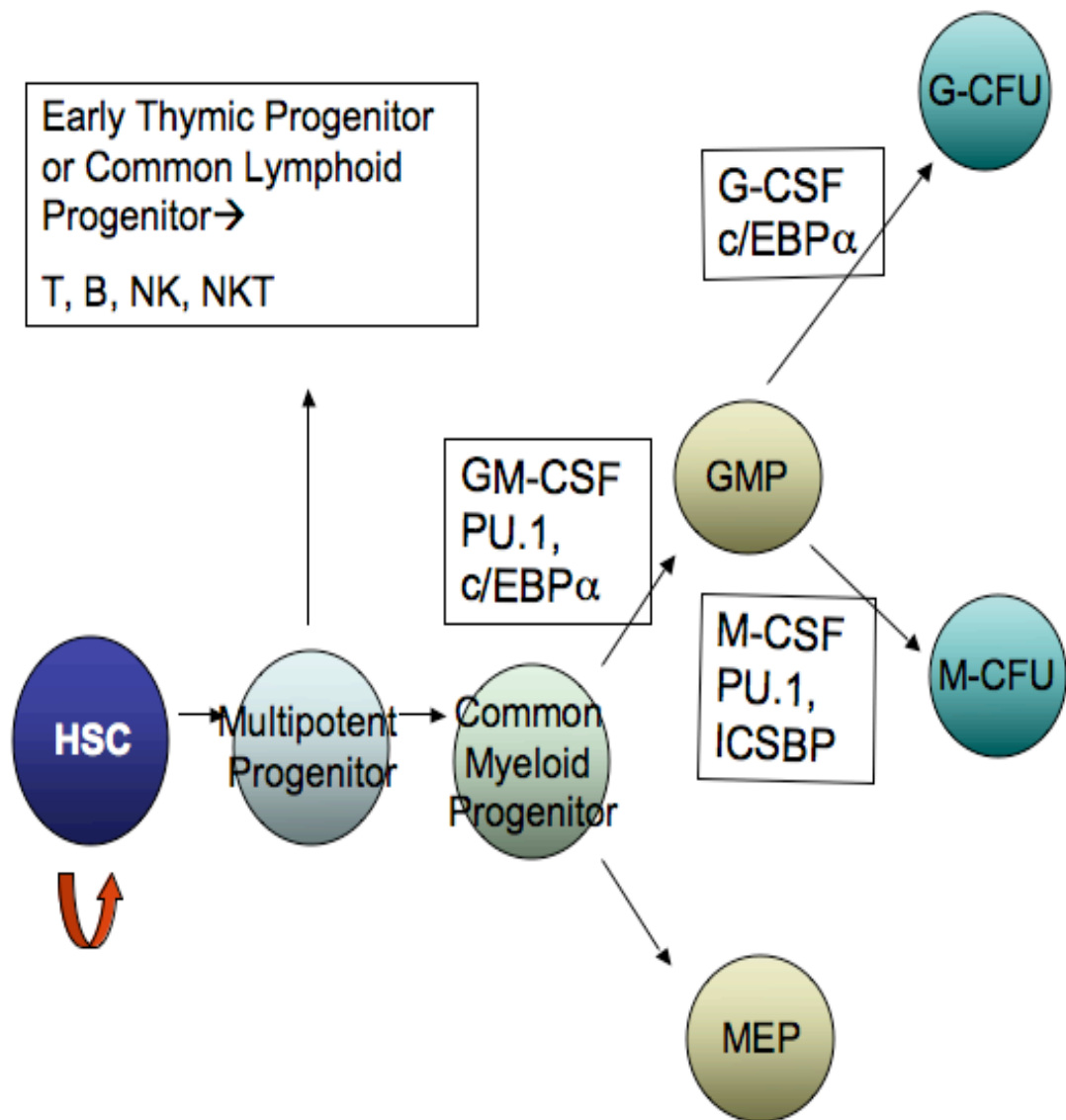


Figure 3. Schematic of myelopoiesis. Myelopoiesis is the process whereby the HSC differentiates into the cells of the myeloid compartment in a branching continuum. It is regulated by cytokines and transcription factors including those shown.

common myeloid progenitors (CMP) (Akashi et al., 2000). CMPs can support erythroid cell differentiation by becoming megakaryocyte-erythroid progenitors (MEP) and subsequently produce megakaryocytes, red blood cells and platelets. On the other hand, CMPs can differentiate to produce granulocyte-macrophage progenitors (GMP), which in turn produce either granulocytes or macrophages and types of dendritic cells (Akashi et al., 2000).

The process of myelopoiesis is regulated by cytokines and by transcription factors (Figure 3). The cytokine granulocyte-macrophage stimulating factor (GM-CSF) promotes the production of GMP, while granulocyte colony stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF) instruct granulocyte and monocyte lineage fate decisions, respectively. The signaling pathways of G-CSF and M-CSF have recently been elucidated. M-CSF activates phospholipase C γ 2 (PLC γ 2), which in turn stimulates extracellular signal-regulated kinase (Erk) activation. Activated Erk leads to transcriptional activity of c/EBP α :c-fos and c-jun heterodimers and Egr-1 which drives monocyte differentiation. On the other hand, G-CSF stimulates signal transducers and activators of transcription 3 (Stat3) activation leading to phosphorylation and increased activity of the phosphatase Src homology 2-containing tyrosine phosphatase-2 (SHP-2) and formation of CCAAT/enhancer binding protein- α (c/EBP α) homodimers, which mediate transcription of genes that promote monocyte differentiation (Jack et al., 2009). Though the exact role of SHP-2 during granulopoiesis is not known, it is known to dephosphorylate and subsequently inhibit the transcriptional activity of interferon regulatory factor-

8 (IRF-8, also known as interferon consensus sequence binding protein, ICSBP), which is a critical regulator of monocyte differentiation (Huang et al., 2006; Tamura and Ozato, 2002). Interestingly, SHP-1 and SHP-2 are subject to oxidative modification that results in inactivation (Chen et al., 2009a; Heneberg and Draber, 2005; Kwon et al., 2005; Weibrecht et al., 2007). Moreover, reactive oxygen species (ROS) generated by lipoxygenases have been implicated in oxidative inactivation of SHP-2 during density dependent inhibition of cell growth (Pani et al., 2000). In chapter 3, I demonstrate that inhibition of SHP-1/2 restores monocyte development and interleukin-12p40 (IL-12p40) production in Alox15 cells, suggesting a pathway by which 12/15-LOX-mediated redox signaling regulates myelopoiesis and inflammation through ICSBP/IRF-8

As described above, cytokines stimulate signaling pathways that result in the activation of transcription factors. The cell fate decision between monocyte and granulocytes are mutually exclusive and pathways that promote one cell fate often inhibit the other. For instance, the transcription factors early growth response factor-1 (Egr-1), Egr-2, ICSBP/IRF-8, NGFI-A binding protein 2 (Nab-2), c-Jun and HoxA10 promote monocyte differentiation and repress granulocyte differentiation while Gfi-1 promotes granulocyte differentiation and inhibits monocyte differentiation (Friedman, 2007; Hock et al., 2003). In addition, the relative levels of transcription factors function as a rheostat to control differentiation; increased levels of PU.1 promote monocyte differentiation while higher levels of the transcription factor C/EBP α promote granulocyte differentiation (Dahl et al., 2003).

ICSBP/IRF-8

The interferon regulatory factor family of transcription factors is known to regulate cell fate decisions and immune regulation (Tamura et al., 2008). One such family member, ICSBP/IRF-8 is an immune cell specific transcription factor important in development of multiple hematopoietic cell types. ICSBP/IRF-8 regulates B cell, dendritic cell and monocyte development and absence of ICSBP/IRF-8 in ICSBP/IRF-8-deficient mice leads to deficiencies of these populations (Lu et al., 2003; Tsujimura et al., 2002). Moreover, the disruption of myeloid cell development in ICSBP/IRF-8-deficient mice results in chronic myelogenous leukemia (CML)-like myeloproliferative disorder that progresses to a blast crisis (Holtschke et al., 1996).

The expression of ICSBP/IRF-8 and other members of the interferon regulatory factor family members are activated by interferon- γ (IFN- γ) signaling through Stat1 (Kanno et al., 1993). ICSBP/IRF-8 consists of 3 domains: DNA binding domain, transcription repressor domain and an IRF-association domain (Sharf et al., 1995). IRF-8 both represses and activates transcription of distinct subsets of genes. ICSBP/IRF-8 activates transcription of genes including neurofibromin (NF-1) (Zhu et al., 2004), p15INK4b (Schmidt et al., 2004), phagocyte oxidase91 (phox91) (Eklund et al., 1998), phox67 (Eklund and Kakar, 1999), B-lymphocyte-induced maturation protein-1 (Blimp-1) (Tamura et al., 2003) and IL-12p40 (Wang et al., 2000) and represses transcription of other genes including bcl-xl (Gabriele et al., 1999), bcl-2 (Burchert et al., 2004), and disabled-2 (Dab2) (Rosenbauer et al., 2002).

ICSBP/IRF-8-transcriptional activity is regulated post-translationally on multiple levels. The gene targeting and transcriptional activity of ICSBP/IRF-8 is conferred in part by its binding to other transcription factors such as PU.1 and other IRF family members (Eklund et al., 1998). The association of ICSBP/IRF-8 with other transcription factors is also believed to regulate whether it functions as a transcriptional activator or repressor. Tyrosine phosphorylation of ICSBP/IRF-8 inhibited its direct binding to DNA but allowed for ICSBP/IRF-8 to bind DNA in association with IRF-1 or IRF-2 (Sharf et al., 1997). On the other hand, the phosphatases SHP-1 and SHP-2 de-phosphorylated ICSBP/IRF-8 causing functional inactivation by inhibiting its association with other transcription factors such as PU.1 (Kautz et al., 2001; Huang et al., 2006). One mechanism whereby oncogenic constitutively active SHP-2 contributes to myeloid leukemia is by decreasing levels of the ICSBP/IRF-8-mediated gene target NF-1, which in turn increases cytokine sensitivity (Huang et al., 2006; Koenigsmann et al., 2009). Moreover, ICSBP/IRF-8 is subject to regulation by proteosomal degradation through Cbl ubiquitin ligase after LPS/IFN γ stimulation (Xiong et al., 2005). Lastly, previous data from our laboratory demonstrated that ICSBP/IRF-8 is also regulated at the level of nuclear accumulation. 12/15-LOX-deficient myeloid splenocytes exhibited similar levels of total but decreased nuclear accumulation of ICSBP/IRF-8. This regulation was mediated in part by PI3-K (Middleton et al., 2006b). In this dissertation, I elucidate that 12/15-LOX regulates ICSBP/IRF-8 nuclear accumulation through a redox-dependent mechanism.

ICSBP/IRF-8 and its target genes are implicated in HSC function.

ICSBP/IRF-8 expression is decreased in aging HSC (Stirewalt et al., 2008), which are known to be functionally compromised (Roobrouck et al., 2008). In addition, ICSBP/IRF-8 represses Dab2 transcription, and ICSBP/IRF-8-deficient cells express high levels of Dab2 (Rosenbauer et al., 2002). Others have shown that Dab2 negatively regulates canonical Wnt signaling by stabilizing axin and the β -catenin degradation complex while inhibiting disheveled (Hocevar et al., 2003; Jiang et al., 2008). Our data demonstrate that Dab2 expression is also increased in Alox15 HSC, which results in decreased canonical Wnt signaling. As described above, canonical Wnt signaling regulates HSC function and may contribute to the defects in Alox15 HSC.

ICSBP/IRF-8 critically promotes monocyte differentiation in conjugation with PU.1 by activating genes that stimulate monocyte differentiation while inhibiting genes that promote granulocyte differentiation (Tamura et al., 2000). ICSBP/IRF-8-deficient mice exhibited defective monocyte differentiation and enhanced granulocyte differentiation that was corrected by retrovirally restoring IRF-8 expression (Tsujiyama et al., 2002). Moreover, ICSBP/IRF-8-deficient mice developed a granulocytic chronic myelogenous leukemia (CML)-like leukemia that progresses into a blast crisis (Holtschke et al., 1996). In addition to its tumor suppressor role in murine leukemia, ICSBP/IRF-8 may also function as a tumor suppressor in human CML. ICSBP/IRF-8 is down-regulated in human CML (Schmidt et al., 1998; Schmidt et al., 2001) and has been shown to antagonize BCR-ABL-induced oncogenesis (Hao and Ren, 2000; Tamura et al., 2003; Burchert et al., 2004). Interestingly, ICSBP/IRF-8 also functions as a

tumor suppressor in other leukemias (Gurevich et al., 2006; Ma et al., 2006; Konieczna et al., 2008; Schwieger et al., 2002).

ICSBP/IRF-8 critically mediates immunity by transcribing IL-12p40 mRNA in antigen presenting cells, dendritic cells and macrophages (Hein et al., 2000) (Jayakumar et al., 2008; Scharton-Kersten et al., 1997). Loss of IL-12p40 gene expression in IRF-8/ICSBP-deficient mice results in susceptibility to myriad of viruses, parasites and bacteria including vaccinia virus, lymphocytic choriomeningitis virus (Holtschke et al., 1996) *Listeria monocytogenes* (Fehr et al., 1997), *Yersinia enterocolitica* (Hein et al., 2000), *Toxoplasma Gondii* (Scharton-Kersten et al., 1997), and *Leishmania major* (Giese et al., 1997). Because of the development of a myeloproliferative disease in ICSBP/IRF-8 deficient mice, less is known about the role of ICSBP/IRF-8 during chronic inflammatory events. Development of a floxed ICSBP/IRF-8 mouse would facilitate further studies into the role of ICSBP/IRF-8 in immunity and chronic inflammation.

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPN) are a result of defective myelopoiesis and are characterized by a clonal expansion of myeloid subsets. Human MPNs include CML, chronic neutrophilic leukemia, polycythemia vera, primary myelofibrosis, essential thrombocythemia, chronic eosinophilic leukemia, mastocytosis, and unclassifiable MPNs (Thiele, 2009).

Defects in transcription factors that regulate myelopoiesis contribute to myeloid leukemias in both mice and humans. For instance, ICSBP/IRF-8

regulates monocyte differentiation and inhibits granulocyte differentiation (Tamura et al., 2000; Tsujimura et al., 2002). In ICSBP/IRF-8-deficient mice, a granulocytic CML-like myeloproliferative neoplasm results (Holtschke et al., 1996). Moreover, during human CML, ICSBP/IRF-8 expression is decreased (Schmidt et al., 1998). Similarly, the transcription factor JunB regulates myelopoiesis and loss of JunB in the myeloid lineage leads to a CML-like MPN (Passegue et al., 2001). Human CML is also associated with decreased JunB expression (Hoshino et al., 2009; Yang et al., 2003). Another example is PU.1, which regulates early myeloid differentiation. PU.1-deficient mice have an early block in myeloid differentiation and disruption of PU.1 results in an acute myeloid leukemia in both mice and humans (Anderson et al., 1998; Mueller et al., 2002).

Alterations in cell signaling can also underlie myeloid leukemogenesis. Loss of NF-1 gene expression in both mice and humans results in hyperactive Ras and cytokine signaling leading to juvenile chronic myelogenous leukemia (Shannon et al., 1994; Kalra et al., 1994; Bollag et al., 1996). Similarly, activating mutations in SHP-2 also stimulates Ras activation resulting in cytokine hypersensitivity and myelogenous leukemia (Niihori et al., 2005; Loh et al., 2004; Schubbert et al., 2005). Polycythemia vera, primary myelofibrosis and essential thrombocythemia represent related MPNs often caused by activating mutations in Jak2 (Levine and Gilliland, 2008). On the other hand, human CML is characterized by presence of a translocation known as the Philadelphia chromosome involving chromosomes 9 and 22. The Philadelphia chromosome is a fusion of Bcr to Abl that results in a constitutively active tyrosine kinase.

Bcr-Abl activates multiple signaling pathways to promote oncogenic signaling including activation of PI3-K and Ras signaling (Ren, 2005). Treatment of CML with the tyrosine kinase inhibitor imatinib has resulted in about 80% clinical and cytogenetic remissions; however, treatment is not curative (Vardiman, 2009).

Since HSCs are the only stage at which self-renewal can occur during normal hematopoiesis often the mutations that lead to leukemia are found in HSCs. For instance, JunB deficiency must be in the HSCs and not in later stages of hematopoiesis for leukemia to result (Passegue et al., 2004). Moreover, deficiency of the tumor suppressor phosphatase tensin homolog (PTEN) in HSCs results in a myeloid leukemia (Yilmaz et al., 2006). In human leukemia, the Bcr-Abl translocation is found in HSCs and drives the chronic phase of disease. However, a secondary mutation develops in GMPs that confers the ability to self-renew and drives the blast crisis disease phase (Jamieson et al., 2004).

Fatty acid metabolism

Metabolism describes the chemical processes in living organisms that support cellular and organismal functions. Fatty acid metabolism describes chemical reactions that involve fatty acids, such as those found in triglycerides and membrane phospholipids. Triglycerides serve to generate energy for the body and are stored in adipose tissue. Meanwhile, the fatty acid components of cell membranes can be directly modified enzymatically or catabolized through the actions of phospholipases to generate free fatty acids. Fatty acid metabolism generates secondary messengers that serve as signaling molecules to regulate cellular processes including vascular homeostasis, platelet aggregation and

inflammatory responses.

During polyunsaturated fatty acid metabolism, lipid substrates such as arachidonic acid (AA) or linoleic acid are released from the cell membranes by phospholipase A₂. The major product AA is then metabolized through the action of oxidative enzymes including cytochrome p450, cyclooxygenases, and lipoxygenases to produce multiple bioactive lipid mediators including eicosanoids, prostaglandins, leukotrienes and lipoxins (Figure 4). These products are generated in a cell-context dependent manner and can signal in both autocrine and paracrine fashions (Harizi et al., 2008). Reactive oxygen species (ROS) are generated as a byproduct of the oxidation step of AA and as the lipid products are further metabolized by glutathione reductase activity (Kim et al., 2008) (Figure 4). The myriad lipid mediators often have opposing functions and hence signaling is dependent on the relative concentration of multiple lipid mediators (Harizi et al., 2008).

Cytochrome p450 and Cyclooxygenases

The cytochrome p450 superfamily is composed of 57 members and is expressed in multiple cell types including liver, adrenal glands, and peripheral blood leukocytes where its expression can be induced by various stimuli (Dy and Schneider, 2004). Cytochrome p450 metabolizes AA to produce hydroxyeicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids (EET). Cytochrome p450 functions in angiogenesis and plays a protective role during myocardial and cerebral ischemia. Moreover, lipid mediators produced by cytochrome p450 have been shown to play an anti-inflammatory role in

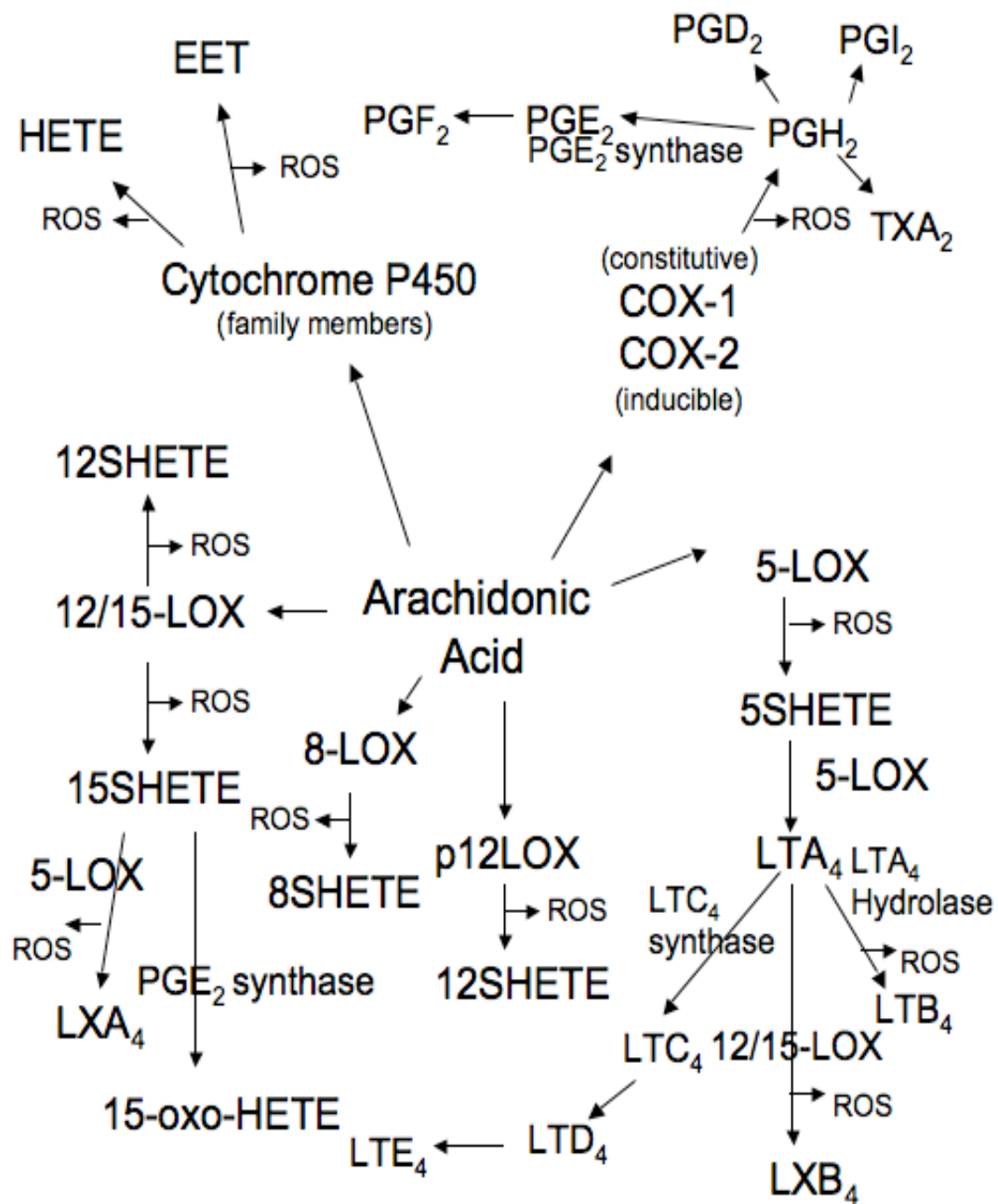


Figure 4. Overview of arachidonic acid fatty acid metabolism. Multiple enzymes including lipoxygenases, cytochrome450 and cyclooxygenaeses oxidize AA to produce many lipid products, some of which are shown here. ROS are generated in multiple reactions.

endothelium and in the kidney (Spector, 2009). Cytochrome p450 lipid products exert their effects extracellularly, through a putative EET receptor or by binding other lipid receptor agonists which are coupled to signaling pathways, and intracellularly by acting directly on ion channels, cell signaling proteins, transcription factors or by incorporation into membrane phospholipids. Interestingly, cytochrome p450 lipid products can have opposing functions. For example, 20-HETE acts on smooth muscle cells as a vasoconstrictor while EET acts as a vasodilator (Kroetz and Zeldin, 2002).

Cyclooxygenases catabolize AA to produce prostaglandins, which exert their pleiotropic effects by binding to multiple G-protein coupled receptors that vary in their secondary signaling pathways and cellular expression (Harizi et al., 2008). Cyclooxygenase-1 (COX-1) is constitutively expressed in most cell types and is highly expressed in endothelium, monocytes, platelets, renal collecting tubules and seminal vesicles. The expression of cyclooxygenase-2 (COX-2) is inducible and restricted to cells involved in reproduction, immunity, renal physiology, neurotransmission, bone resorption and pancreatic secretion. COX-2 is induced by multiple proliferative and inflammatory stimuli including tumor necrosis factor- α , lipopolysaccharide (LPS) and IL-1 and decreased by glucocorticoids and anti-inflammatory stimuli (Smith et al., 2000). Nonsteroidal anti-inflammatory drugs, such as aspirin and ibuprofen, target the COX isoforms as a treatment for pain, fever, and inflammation (Rouzer and Marnett, 2009). COX-2 is pro-inflammatory in peripheral tissues and participates in inflammatory diseases such as cancer and in rheumatoid arthritis (Obukowicz and

Ornberg, 1999; Rouzer and Marnett, 2009; Tuynman et al., 2008). COX-2 specific inhibitors have been developed to treat chronic inflammation but its use is limited due to cardiovascular toxicity, which is associated with decreased PGI₂ production in the blood vessel wall (Rho et al., 2009; Rouzer and Marnett, 2009). On the other hand, COX-2 can also decrease inflammation in a cell-context dependent manner. For instance, COX-2 through prostaglandin E₂ (PGE₂) can decrease inflammation in secondary lymphoid organs by decreasing IL-12p40 production through an IL-10 dependent mechanism in dendritic cells (DC), inducing forkhead box P3⁺ (FoxP3) regulatory T cells, suppressing natural killer (NK) cell IFN γ production and inducing B cell immunoglobulin (Ig)-class switching (Harizi et al., 2008; Harizi and Gualde, 2006).

Lipoxygenases

Lipoxygenases are a diverse class of oxidative enzymes that catalyze oxygenation of AA and linoleic acid to generate numerous hydroperoxy-eicosatetraenoic acid (HpETE) intermediates (Conrad, 1999). 5-Lipoxygenase (5-LOX) is an enzyme primarily expressed in leukocytes that catalyzes the conversion of AA to 5-HpETE and subsequently catalyzes the reaction of 5-HpETE to generate leukotriene A₄. 5-LOX is bound by 5-lipoxygenase activating protein that enhances its substrate binding. Leukotriene A₄ is subsequently converted to leukotriene C₄ (LTC₄) in cells that express LTC₄ synthase such as mast cells, eosinophils, and endothelial cells or converted to leukotriene B₄ in cells that express LTA₄ hydrolase such as neutrophils and monocytes. LTC₄ can be further metabolized to produce LTD₄, and LTE₄. Leukotrienes are

released from the cell by protein transporters and signal by binding G-coupled protein receptors BLT₁, BLT₂, cysLT₁, cysLT₂ (Peters-Golden and Henderson, 2007). The leukotrienes produced depend on cellular context, and their signaling depends on the expression of receptors on neighboring cells. Leukotrienes stimulate leukocyte recruitment of multiple cell types to inflammatory sites and their subsequent activation (Islam et al., 2006; Tager et al., 2000; Lee et al., 1999; Shin et al., 2006; Taube et al., 2006). Leukotrienes also increase Th2 inflammatory responses, vascular permeability, and bronchoconstriction and contribute to the pathogenesis of asthma, cardiovascular disease, arthritis and cancer (Hyde and Missailidis, 2009; Peters-Golden and Henderson, 2007; Woszczek et al., 2005; Williams and Spector, 2009). Moreover, inhibitors of 5-LOX and cysLT1 are used in the treatment of asthma (Israel et al., 1996; Schmidt et al., 2005; Van Schoor et al., 1997).

5-LOX in concert with 15-lipoxygenase (15-LOX) or 12-lipoxygenase, generates lipoxin A₄ and lipoxin B₄. Not only does lipoxin production decrease leukotriene production, generally, the actions of lipoxins function to oppose the actions of leukotrienes (Serhan, 2005). Lipoxins control the resolution of inflammation by stimulating anti-inflammatory pathways such as inhibiting neutrophil infiltration, acting as a chemoattractant for macrophages and stimulating their uptake of apoptotic cells, and decreasing production of pro-inflammatory cytokines such as IL-1 and IL-8 (Papayianni et al., 1996; Godson et al., 2000; Lee et al., 1989; Bonnans et al., 2002; Machado et al., 2008; Maddox et al., 1997; Sodin-Semrl et al., 2000; Wu et al., 2005). These actions play a

role in the resolution of inflammatory events such as airway inflammation, asthma, arthritis, and gastrointestinal disease (Kronke et al., 2009; Serhan, 2005; Janakiram and Rao, 2009; Levy et al., 2002; Wallace et al., 2005).

12/15-Lipoxygenase

In the process of metabolizing polyunsaturated fatty acids, 12/15-lipoxygenase (12/15-LOX) generates multiple products. 12/15-LOX introduces molecular oxygen into AA and linoleic acids to produce bioactive labile lipid intermediates such as 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HpETE), 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HpETE) and 13(S)-hydroperoxyoctadecadienoic acid (13(S)-HpODE). These intermediates are rapidly reduced by glutathione reductase to release ROS and produce additional bioactive lipid metabolites, including 12(S)-HETE, 15(S)-HETE, 13(S)-HODE, lipoxins, and hepoxilins (Kuhn and O'Donnell, 2006) (Figure 5). 12/15-LOX can also oxidize membrane-bound fatty acids directly to produce additional fatty acid products. Moreover, novel products of 12/15-LOX are still being defined (Wei et al., 2009).

Humans have multiple 12/15-lipoxygenases, including leukocyte-type 12-lipoxygenase, 15-lipoxygenase-1 and 15-lipoxygenase-2 all of which have the ability to produce 12(S)-HETE as well as 15(S)-HETE albeit in different ratios. 15-Lipoxygenase-1 is expressed in reticulocytes, leukocytes, bronchial epithelial cells and carcinoma cells while 15-lipoxygenase-2 is not expressed in peripheral leukocytes but rather in liver, spleen, kidney, intestines, testis, ovary, muscle, heart and brain. 15-Lipoxygenase-1 and 15-lipoxygenase-2 are co-

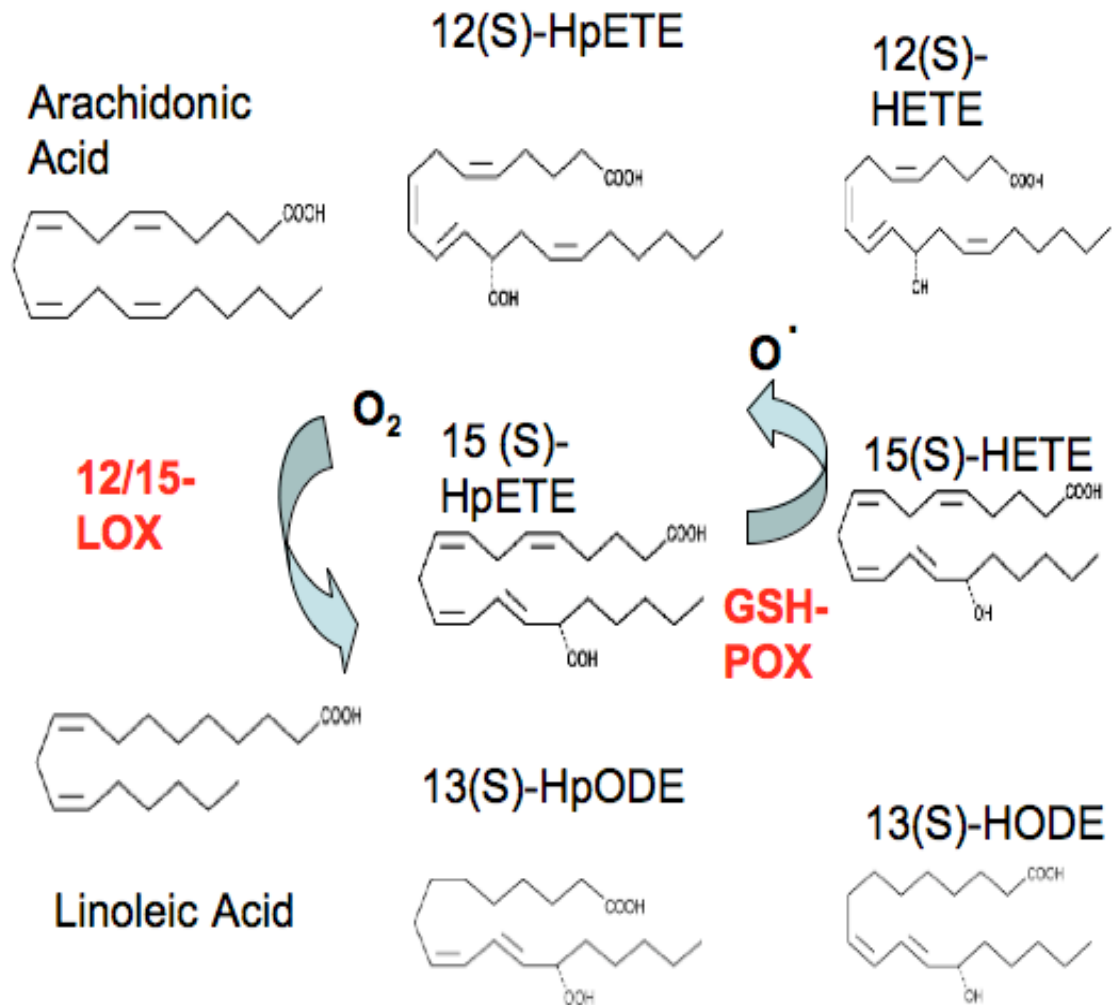


Figure 5. 12/15-LOX-Mediated Fatty Acid Metabolism. 12/15-LOX functions in part by adding a molecular oxygen onto the polyunsaturated fatty acid substrates arachidonic and linoleic acids to produce 12(S)-hydroperoxyeicosatetraenoic acid (12(S)-HpETE), 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HpETE) and 13(S)-hydroperoxyoctadecadienoic acid (13(S)-HpODE). These intermediates are unstable and rapidly metabolized through glutathione peroxidase to release reactive oxygen species and additional bioactive lipid metabolites, including 12(S)-HETE, 15(S)-HETE, 13(S)-HODE.

expressed in bronchial epithelial cells (Chanez et al., 2002). Leukocyte-type 12-lipoxygenase is expressed in leukocytes, liver, lung, and endothelial cells (Funk et al., 1992). On the other hand, mice possess a single leukocyte-type 12/15-lipoxygenase, which produces 12(S)-HETE and 15(S)-HETE at a ratio of 3:1 (Chen et al., 1994). Therefore, murine 12/15-LOX and human 12- and 15-lipoxygenases differ in lipid mediator production. Some of the effects of murine 12/15-LOX are analogous to human leukocyte 12-lipoxygenase (Mullin et al., 2007) while others map to 15-lipoxygenase-1 (Kelavkar et al., 2004). Therefore, in order to elucidate the relevant human counterpart, it is important to understand the products of murine 12/15-LOX that mediate its biological effects. Platelet-type 12-lipoxygenase and epidermal-type 12-lipoxygenase are additional lipoxygenases found in humans and mice but are less related to leukocyte-type 12-lipoxygenase and 15-lipoxygenase-1 both by sequence and function.

12/15-Lipoxygenase products can activate multiple signaling pathways resulting in activation of transcription factors including peroxisome proliferator-activated receptor γ , IRF-8, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) that then modulate gene transcription (Huang et al., 1999; Middleton et al., 2006a) (Figure 6). 12(S)-HETE and 15(S)-HETE both activate protein kinase C (PKC) while 15(S)-HETE and 13(S)-HODE activate the mitogen activated protein kinase (MAPK) pathway. Products of 12/15-LOX can also be incorporated into cellular membranes to regulate cellular functions (Takata et al., 1994; Kuhn and Brash, 1990; van Leyen et al., 1998). 12/15-LOX lipid mediator signaling events have pleiotropic effects on myriad cell types.

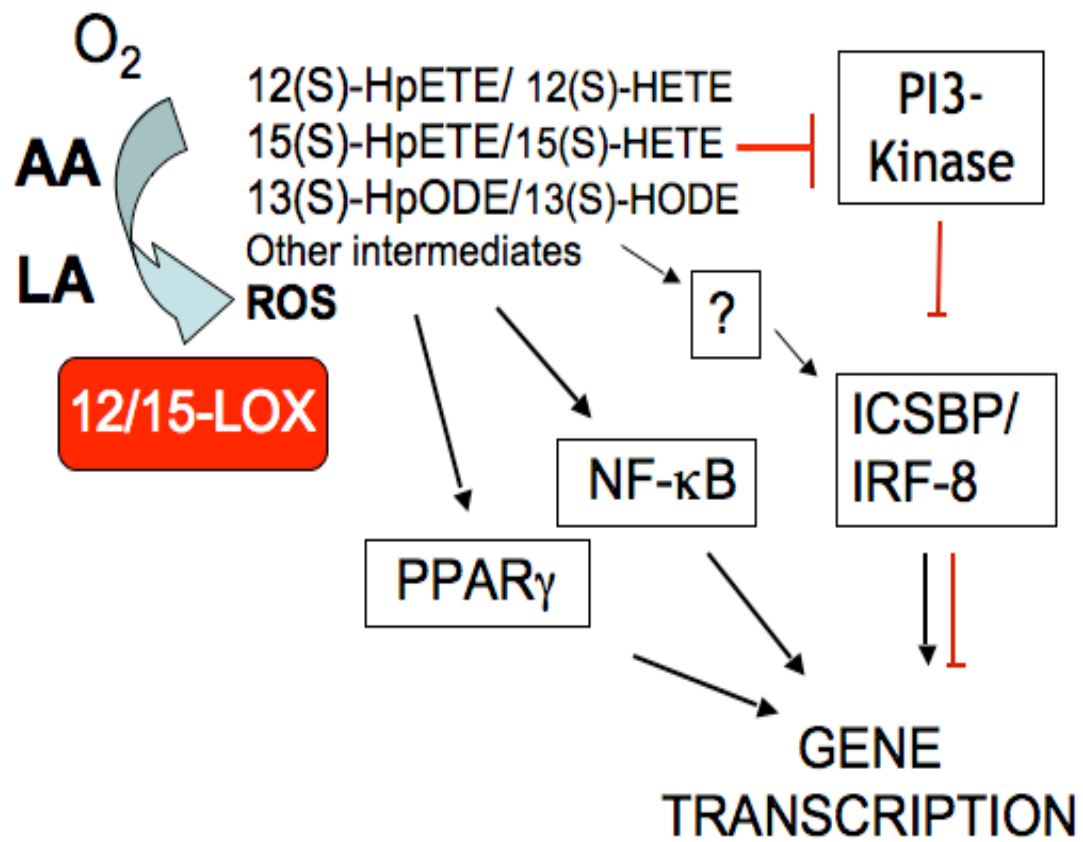


Figure 6. 12/15-Lipoxygenase products initiate multiple signaling pathways. The products of 12/15-LOX can initiate multiple signaling pathways regulating gene transcription by a PI3-K-dependent and possibly independent regulation of the transcription factor ICSBP/IRF-8, and activation of NF-κB and PPAR γ transcription factors.

Notably, 12/15-LOX regulates the pathogenesis of atherosclerosis, psoriasis, arthritis, glomerular nephritis, and airway inflammation (Conrad, 1999).

Reactive Oxygen Species

ROS are another important product of fatty acid metabolism. Cellular ROS, such as superoxide O_2^- , hydrogen peroxides H_2O_2 , hydroxyl radical OH^\cdot and hypochlorous acid $HOCl$, are produced through the actions of nicotinamide adenine dinucleotide phosphate oxidases (NAPDH oxidases), mitochondria and fatty acid metabolism (Ghaffari, 2008; Haddad, 2002a). ROS regulate multiple signaling processes. For instance, ROS play a paramount role during phagocyte killing of bacteria in a process known as the oxidative burst. ROS can also be highly toxic to cells by causing oxidative stress resulting in cellular and tissue damage through oxidative modification of cellular components and activation of apoptosis. Therefore, the levels of cellular ROS are tightly controlled by the presence of anti-oxidants. Cellular anti-oxidants are present in various forms including reducing agents such as vitamin E, vitamin C, glutathione and including enzymes such as catalase, superoxide dismutase and peroxidases. For example, superoxide is catalyzed by superoxide dismutase to produce hydrogen peroxide H_2O_2 , which is further metabolized by catalase to produce molecular oxygen and water (Ghaffari, 2008). Inhibition of anti-oxidants in the cell can result in oxidative stress (Jones, 2008).

ROS mediate redox reactions, which is the shuffling of electrons in chemical reactions. Redox signaling is always occurring within the cell in homeostatic conditions to regulate essential cellular functions. For instance,

redox signaling in the electron transport chain in mitochondria generates the majority of energy in the body as adenosine triphosphate (Hancock, 2009).

Redox signaling also regulates protein function. Cysteines within proteins contain a thiol residue that is susceptible to oxidative modification, which can result in the formation of disulfide bonds if two cysteine residues are in close proximity.

Oxidative modification of a protein residue can have multiple effects on protein function. If the residue is in a catalytic site of an enzyme, oxidative modification can cause functional inactivation. If the residue is in an allosteric site, oxidative modification can alter protein conformation and function as a rheostat for protein activity. Moreover, oxidative modification of proteins can alter protein binding and result in decreased or increased binding to substrates such as DNA, membranes, cytoskeleton or other proteins (Jones, 2008).

In theory, redox signaling can regulate any protein with an accessible cysteine residue. There is an average of 1.9 cysteine residues per protein in humans. Therefore, redox signaling could theoretically regulate many proteins. Because of the need for cysteine residues to be accessible it is likely that only 10-20% of human proteins are in fact directly regulated by redox signaling (Jones, 2008). Many protein targets of redox reactions have been identified including receptors such as $\alpha\text{IIb}\beta\text{3}$ integrin, phosphatases such as SHP-1/2, PTEN and protein phosphatase-1 α (PP1 α), cytoskeletal components such as actin and signal transducers such as caspases, ras-related C3 botulinum toxin substrate 1 (Rac1), I κ B kinase (IKK) and kelch like-ECH-associated protein 1 (Keap1). Production of ROS mediates modulation of multiple signaling

pathways including activation of p-53, Ras, PI3-K and p38 MAPK signaling and modulation of gene transcription by NF κ B, nuclear factor erythroid-derived-like 2 (Nrf2), Egr-1 (Haddad, 2002a; Hainaut and Mann, 2001; Iiyama et al., 2006; Naughton et al., 2009; Pan et al., 2009). ROS signaling results in cellular functions such as proliferation and secretion of inflammatory cytokines (Iiyama et al., 2006) (Haddad, 2002b). On the other hand, hypoxia can result in migration of macrophages and DC and activation of transcription factors including HIF1 α , HIF2 α , activator protein-1 (AP-1), activating transcription factor-2/5 and Fos homolog B (Bosco et al., 2008; Haddad, 2002a). This dissertation elucidates a novel target downstream of redox signaling, the transcription factor IRF-8 and implicates the redox sensitive phosphatase SHP-1/2 in this regulation.

Alox15 mice

Mice deficient in 12/15-LOX, Alox15 mice, have been developed by targeted disruption of exon 3 to generate an in-frame stop codon prior to the catalytic domain (Sun and Funk, 1996). Alox15 mice have been extensively studied in the context of inflammation. For instance, Alox15 mice were protected against atherosclerosis (Cyrus et al., 2001; George et al., 2001). This protection was associated with decreased ox-LDL production in Alox15 macrophages (Sun and Funk, 1996) and decreased IL-12p40 production in Alox15 atherosclerotic lesions (Zhao et al., 2002). Alox15 mice were also protected against inflammation caused by allergic asthma (Andersson et al., 2008; Hajek et al., 2008) and acute lung injury (Zarbock et al., 2009). The attenuated inflammation was associated with decreased levels of the 12/15-LOX product 12(S)-HETE

(Zarbock et al., 2009). However, Alox15 mice exhibited exacerbated inflammation in a tumor necrosis factor transgenic model of arthritis (Kronke et al., 2009), which was associated with decreased levels of the anti-inflammatory 12/15-LOX-mediated product, LXA₄. Therefore, the signaling downstream of 12/15-LOX depends on the production and context of specific 12/15-LOX-generated products.

12/15-LOX promotes IL-12p40 production in macrophages. Alox15 peritoneal macrophages but not bone marrow derived dendritic cells exhibited decreased levels of IL-12p40 in response to LPS and IFN- γ stimulation. This defect was associated with decreased levels of IRF-8 and NF- κ B binding to the IL-12p40 promoter (Middleton et al., 2006a). Others also demonstrated that 12/15-LOX contributes to inflammatory gene expression in macrophages (Dioszeghy et al., 2008). 12/15-LOX regulation of IL-12p40 and inflammatory gene transcription in macrophages has implications for inflammatory diseases in which macrophages play a predominant role. As stated previously, 12/15-LOX-dependent IL-12p40 production contributed to the inflammatory milieu and disease progression of atherosclerosis (Zhao et al., 2002). Moreover, Alox15 mice were defective in immunity to chronic toxoplasma gondii infection but not during acute infection (Middleton in press) highlighting the selective role of 12/15-LOX and macrophages during chronic inflammation. In this dissertation, I will demonstrate that 12/15-LOX regulates IL-12p40 production in macrophages at least in part through ROS signaling.

MPN in Alox15 mice

In the course of studying Alox15 mice in the context of chronic inflammation, our lab made the unexpected finding that Alox15 mice exhibit increased mortality due to development of a CML- like MPN in about 10-15% of the mice over the course of a year. The majority of Alox15 mice remained asymptomatic and exhibited splenomegaly, basophilia, and an increased percentage of granulocytes in blood and spleen. Alox15 Gr1⁺ splenocytes exhibited increased proliferation and decreased cell death compared to wild-type. Addition of the 12/15-LOX product 12(S)-HpETE increased cell death in both Alox15 cells and the human K562 CML cell line (Middleton et al., 2006b). Asymptomatic Alox15 myeloid cells also exhibited increased phosphorylation of the tyrosine kinase Akt, and decreased nuclear accumulation and transcriptional activity of tumor suppressor IRF-8. Meanwhile, the moribund mice exhibited a MPD characterized by massive splenomegaly, accumulated granulocytes in the bone marrow, spleen, blood and skin and the presence of myeloid blasts. This phase of disease was associated with the loss of IRF-8 gene expression (Middleton et al., 2006b). Because a proportion of Alox15 mice develop a MPN, I hypothesized that 12/15-LOX regulates myelopoiesis. In this dissertation, I will demonstrate that 12/15-LOX does indeed regulate myelopoiesis and elucidate the mechanisms whereby this regulation occurs.

12/15-LOX in Hematopoiesis

Although the relationship between fatty acid metabolism and inflammation in mature leukocytes has been extensively studied, little is known about the contributions of fatty acid metabolism to hematopoietic development. During

erythroid development in rabbits and humans, the 12/15-LOX products 15(S)-HETE and 13(S)-HODE modulate membrane degradation, an important step in erythroid differentiation (Kuhn and Brash, 1990; van Leyen et al., 1998). However prior to my studies, the role of 12/15-LOX in murine erythroid development was unknown.

Little was previously known about the role of 12/15-LOX and its products in myeloid development. Addition of 12/15-LOX lipid products 12(S)-HETE and 15(S)-HETE to cultures of human CD34⁺ cells decreased the numbers of granulocyte-macrophage colonies in methylcellulose assays (Desplat et al., 2000). However the role of 12/15-LOX in granulocyte and monocyte differentiation has remained unexplored. Data presented in this dissertation demonstrate that 12/15-LOX promotes monocyte development.

As stated earlier, previous studies in our lab demonstrated that 12/15-LOX is a novel suppressor of MPN (Middleton et al., 2006b). Approximately 15% of Alox15 mice develop a severe MPN over the course of a year. Moreover, the lipid mediators of 12-lipoxygenase are reduced in human CML (Stenke et al., 1991; Stenke et al., 1987; Takayama et al., 1983) implicating 12/15-LOX in the regulation of human disease as well. Although 12/15-LOX acts as a suppressor of myeloid leukemogenesis, its role in normal myeloid development was not previously known.

ICSBP/IRF-8, a critical regulator of lineage specification and immunity, is regulated by 12/15-LOX. Our lab previously demonstrated that IRF-8 DNA binding and nuclear accumulation were compromised in Alox15

macrophages and splenocytes (Middleton et al., 2006a; Middleton et al., 2006b). This resulted in decreased IL-12p40 transcription in Alox15 macrophages (Middleton et al., 2006a) and in a CML-like myeloproliferative disorder in Alox15 mice (Middleton et al., 2006b). IRF-8 was shown in these studies to be regulated by 12/15-LOX in part through phosphoinositide 3-kinase (PI3-K). PI3-K activity as measured by pAKT levels was increased in Alox15 BM-derived cells and inhibition of PI3-K resulted in increased nuclear accumulation of IRF-8 and restored gene transcription (Middleton et al., 2006b). Because 12/15-LOX regulates IRF-8 in mature myeloid cells, I hypothesized that 12/15-LOX also regulates IRF-8 in immature myeloid cells. Moreover, because IRF-8 is important to the development of multiple hematopoietic lineages, I hypothesized that 12/15-LOX may also regulate hematopoiesis by promoting ICSBP/IRF-8 nuclear accumulation and transcriptional activity.

ROS in Hematopoiesis

ROS, which are functional by-products of 12/15-LOX activity (Conrad, 1999), are known to regulate HSC function. Low levels of ROS maintained through interactions in the osteoblastic HSC niche are critical for LT-HSC function (Jang and Sharkis, 2007). The oxidative state of HSCs is also regulated in part by NADPH-oxidases (Piccoli et al., 2007). However, the role of lipoxygenase-generated ROS has not yet been defined in HSC.

Increased levels of ROS can signal through p38 MAPK to cause defective HSC quiescence and self-renewal. Treatment of normal bone marrow with buthionine sulfoximine (BSO), which increases ROS by inhibiting synthesis

of the anti-oxidant glutathione, increased p38 MAPK specifically in HSC and inhibited the ability of HSC to reconstitute lethally irradiated mice (Ito et al., 2006). Levels of ROS increased in HSC during serial transplantations and treatment with the anti-oxidant N-acetylcysteine (NAC) or with a p38 MAPK inhibitor extended the self-renewal ability of serial reconstituted HSC (Ito et al., 2006). Deletion of the DNA repair enzyme ataxia telangiectasia mutated (ATM) increased levels of cellular ROS, decreased quiescence and increased p38 MAPK activation in the HSC. This resulted in a defect of ATM-deficient HSC to reconstitute lethally irradiated mice. Treatment with a p38MAPK inhibitor (SB203580) restored HSC quiescence and function (Ito et al., 2006). ROS in HSC were also increased when members of the forkhead box subgroup O (FoxO) transcription factor family (FoxO1/3/4) were deleted. Similar to ATM-deficient HSC, FoxO-deficient HSC exhibited increased levels of ROS, decreased quiescence and a defect in the ability to reconstitute lethally irradiated mice. Treatment with the anti-oxidant NAC restored HSC quiescence and function (Tothova et al., 2007). The perturbed HSC function associated with FoxO deletion was also associated with activation of p38 MAPK. Moreover, FoxO3a-deficient HSC exhibited similar defects as FoxO1/3/4-deficient HSC. FoxO3a-deficient HSC exhibited increased activation of p38MAPK and treatment with a p38MAPK inhibitor restored HSC function in this mouse model (Miyamoto et al., 2007).

The tuberous sclerosis complex (TSC)-mammalian target of rapamycin (mTOR) pathway also functions in HSC to suppress ROS production and

promote HSC quiescence and self-renewal. When TSC was conditionally deleted in hematopoietic cells, the HSC exhibited increased proliferation and defective self-renewal. These defects were accompanied by an increase of ROS in the HSC in part by increasing mitochondrial biogenesis. Treatment with the anti-oxidant NAC restored HSC function (Chen et al., 2008). Hence, accumulation of ROS negatively impacts HSC function and self-renewal in multiple mouse models. In this dissertation, I demonstrate that Alox15 HSC exhibit increased basal levels of ROS but decreased levels of ROS in response to AA that is likely 12/15-LOX-dependent. The increased basal levels of ROS in Alox15 HSC may contribute to their defective self-renewal and quiescence.

ROS may also have effects on myeloid cell fate decisions. ROS mediate cytokine receptor signaling, including in response to GM-CSF. Addition of ROS in the form of hydrogen peroxide increased tyrosine phosphorylation of cytokine receptors and signal transducers while addition of anti-oxidants had the converse effect (Iiyama et al., 2006; Sattler et al., 1999). Moreover, ROS generated by dominant negative NF- κ B inhibits granulocyte differentiation, which was restored by addition of an anti-oxidant (Nakata et al., 2004). However, this study did not examine the effects on mature monocyte development. In this dissertation, I demonstrate that ROS mediate monocyte development in Alox15 mice.

Summary

In summary, in the following chapters I will describe novel functions for 12/15-LOX in hematopoietic development. Because a percentage of Alox15 mice develop a myeloproliferative disorder, I hypothesized that 12/15-LOX

regulates hematopoietic development. To test this hypothesis, I characterized hematopoietic subsets in wild-type and Alox15 mice. I found that 12/15-LOX regulates HSC function and monocyte development. For my dissertation, I elucidated the mechanisms whereby this regulation occurs. An overview of my dissertation is shown in Figure 7.

Hypothesis: 12/15-LOX regulates hematopoiesis

- Aim 1: Characterize hematopoietic differentiation in Alox15 mice
- Aim 2: Determine the mechanisms whereby 12/15-LOX regulates hematopoietic stem cell function
- Aim 3: Determine the mechanisms whereby 12/15-LOX regulates myelopoiesis

Figure 7. Overview of hypothesis and aims.

EXPERIMENTAL METHODS

Mice- C57BL/6 (B6) and Alox15 mice (backcrossed to B6 11 generations) purchased from Jackson Laboratories, were housed and bred in the animal facility of the Wistar Institute. Mice were used between 8-10 weeks of age prior to the onset of MPD except where explicitly stated. Congenic B6.SJL mice were obtained from Taconic or NCI. All animal procedures were approved by Wistar Institute institutional care and animal use committee.

Hematologic analysis- Whole blood obtained via submandibular bleeding diluted with 1% PBS/PBS with 5 μ L of 0.5 M EDTA. Blood was analyzed on an Advia2120 hematology analyzer in the mouse mode (Siemens Healthcare Diagnostics).

Fetal liver isolation-Two female mice and 1 male mouse were placed in a cage overnight. Mice were then separated by sex and pregnant mice were sacrificed 14 days later to isolate fetal liver from day 14.5 embryos.

Macrophage isolation-To generate bone marrow derived macrophages, bone marrow from wild-type and Alox15 mice were isolated from the long bones. Cells were subject to RBC lysis using ammonium chloride and were cultured at 1×10^6 cells per ml, in 10 ml of 10% L292 supernatant in RPMI containing 10% fetal calf serum (FCS), 50 μ M β -Mercaptoethanol, 1% penicillin, streptomycin and fungizone on untreated 10cm² tissue culture plates. After 5 days, non-adherent cells were washed away and purity was checked by flow cytometry. Cells were at least 85-90% macrophages by F4/80⁺ staining and flow cytometric analysis. To isolate thioglycolate-elicited macrophages, B6 and Alox15 mice were

injected intraperitoneally with 2ml sterile 3% Brewer's thioglycollate broth (Sigma-Aldrich). Four days later macrophages were isolated peritoneal cavity with PBS. Cells were plated for 2 hours to overnight in 10% FCS in RPMI containing 50 μ M β -Mercaptoethanol, 1% penicillin, streptomycin and fungizone. Non-adherent cells were washed away. The remaining cells were at least 90% pure by F4/80⁺ staining and flow cytometric analysis.

Flow cytometry- Single cell suspensions were prepared from thymus, spleen, fetal liver and BM isolated from tibia and fibulas. RBC were lysed using ammonium chloride except for erythroid progenitor analysis.

Immunocytochemistry reagents were obtained from the following sources: cKit, Invitrogen; CD34, CD45.1, Biolegend; CD3, IgM, Gr1, streptavidin-conjugates, BD Bioscience; all others, eBioscience. Differentiated cells were depleted with a cocktail of antibodies directed against: Gr-1, B220, CD3, IL-7R α , Ter119, NK1.1, Mac1 and CD11c; anti-IL7R α was omitted when analyzing CLP, ETP, DN2 and DN3. Cells were resuspended for staining in 1%BSA/PBS and blocked for nonspecific staining using murine IGG (Jackson ImmunoResearch) for 5 minutes room temperature before addition of antibodies. Cells were stained for 30 minutes at 4C and washed with PBS. Secondary antibody staining was in phosphate buffered saline (PBS) for 30 minutes at 4C. Cells were analyzed by flow cytometric analysis on a FACS Calibur or LSR II using Flowjo software (Treestar).

Cell cycle analysis- Bone marrow was isolated and stained at 1x10⁶ cells/ml in 2% FCS RPMI with 10 μ g/ml Hoechst 33342 at 37C incubator for 45 minutes

followed by addition of 300 ng/ml PyroninY (Sigma-Alrich) for an additional 45 minutes. Cells were then washed with cold PBS and kept at 4⁰C for subsequent multicolor flow cytometric analysis to identify progenitor populations. In some experiments, bone marrow was treated for 45 minutes or overnight with increasing concentrations of 12(S)-HETE or 15(S)-HETE (Cayman Chemical).

AnnexinV and TUNEL staining- For AnnexinV staining, cells were stained for multicolor flow cytometry prior to incubation with 5 µl AnnexinV-FITC (Invitrogen) in 100 µl AnnexinV staining buffer for 10 minutes at room temperature after which 400 µl additional staining buffer was added.

In situ staining kit for Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was obtained from Roche. Staining was performed according to manufacturer's protocol. Treatment with DNase was used as a positive control while fluorescence minus one (FMO) (without fluorescein-dUTP) was used as a negative control.

Bone marrow reconstitution and 5-FU assays -Recipient mice were irradiated with 1000 rads of γ irradiation from a Cesium J. L. Sheppard Mark irradiator. Mice were transplanted with 1.8x10⁶ test: 2x10⁵ competitor BM cells (9:1), 2x10⁵:2x10⁵ competitor BM cells (1:1), 1x10⁶ E14.5 fetal liver cells: 0.5x10⁶ competitor BM cells (2:1) and 0.5x10⁶ E14.5 fetal liver cells: 0.5x10⁶ competitor BM cells (1:1) for competitive reconstitution assays and 1x10⁶ BM cells for noncompetitive and serial reconstitution assays. Mice were injected i.p. with 200 mg/kg 5-FU (Roche) to determine self-renewal ability.

Cell separation-Lin⁻cKit⁺ cells were obtained using MACS separation kit

(Miltenyi-biotech). Briefly, cells were resuspended in 40 μ L MACS Buffer (0.5% BSA 2 mM EDTA PBS pH 7.2) per 10^7 cells and 10 μ L of lineage antibodies were added per 10^7 cells. Cells were incubated for 10 minutes at 4°C prior to addition of 30 μ L MACS buffer per 10^7 cells and 20 μ L anti-biotin microbeads. Cells were then incubated for an additional 15 minutes prior to washing with PBS. Cells were resuspended with 500 μ L MACS buffer per 10^8 cells and the negative fraction was obtained on LS columns. For some experiments, cKit microbeads were used subsequent to the Lin⁻ purification. Cells were counted and resuspended in 80 μ L MACS buffer per 10^7 cells and 20 μ L cKit microbeads were added per 10^7 cells. Cells were incubated for 15 minutes prior to washing and the positive fraction was collected on MS columns. In all experiments, purity was checked using flow cytometric analysis and determined to be at least 90% pure.

Immunoblotting- Nuclear extracts were prepared using NucleoBuster kit (EMD Biosciences) from Lin⁻cKit⁺ cells pooled from 3 mice and from bone marrow-derived macrophages. MPER was used to obtain total (cytoplasmic and nuclear) cell lysates. Lysates were normalized to total protein using Bradford assay (Pierce) and resolved by 7.5% SDS PAGE, transferred to PVDF membranes, and immunoblotted with antibodies specific for ICSBP/IRF-8, Dab2, Rb, 12/15-LOX (Santa Cruz Biotechnology) and β -catenin (BD Bioscience). Peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch.

ROS and lipid analysis- ROS levels were analyzed on LSK enriched by cell sorting and on BMM by loading with 10 μ M CM-H₂DCFDA (Invitrogen) in the presence (LSK) or absence (LSK, BMM) of 10 μ M AA (Cayman Chemical)

(LSK) for 30 min at 37°C. Cells were then analyzed by flow cytometry. For lipid analysis, BM, Lin⁻ enriched cells and BMM were loaded for 30 minutes with 10 µM AA (Cayman Chemical), the supernatants were extracted and analyzed by stable isotope dilution normal phase chiral liquid chromatography coupled with electron capture atmospheric pressure chemical ionization/mass spectrometry.

Cell sorting and quantitative-PCR-BM cells from 3-5 mice were pooled, stained and sorted on an Aria cell sorter at the University of Pennsylvania or Wistar Institute flow cytometry facility. mRNA was isolated using RNeasy microRNA kit (Qiagen) and cDNA synthesized using reverse transcriptase. Quantitative real time PCR was performed with Syber green master mix in an ABI 7000 cycler (Applied Biosystems) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Primer sequences were designed using Primer Express (Applied Biosystems).

Primer Sequences

Gapdh (forward) 5'-G A C G G C C G C A T C T T C T T G T and (reverse) 5'-C A C A C C G A C C T T C A C C A T T T T-3'; β- actin, (forward) 5'-T C A G C A A G C A G G A G T A C G A T G -3' and (reverse) 5'-A A C A G T C C G C C T A G A A G C A C T T-3'; Icsbp/Irf8 (forward) 5'T G G G C A G T T T T T A A A G G G A A G T T-3' and (reverse) 5'-A C A G C G T A A C C T C G T C T T C C A -3'; 12/15- Lox (forward) 5'-A C C C C A C C G C C G A T T T T and (reverse) 5'-A G C T T C G G A C C C A G C A T T T; Dab2(forward) 5'-G G C C A C A C C G C C T C A A- 3'(reverse) 5'-A G C G T C A C T G G G A A T G T C C T T-3'; Nf1 (forward) 5'-G G T A T G G A C A C A G G A C C T C T A G G A T-3'

and (reverse) 5'-C T G C G G G T A C C A T G A A G C A-3'; Blimp1 (forward) 5'-G G A G G A T C T G A C C C G A A T C A-3' and (reverse) 5'-T C C T C A A G A C G G T C T G C A T T G-3'; p15Ink4b(forward) 5'-T G G G T G G G T G C A G T C A G T A C-3' and (reverse) 5'-T C C C A T T T A G C C T G T G G A T C A-3'; Hes-1 (forward) 5'-T C A G C G A G T G C A T G A A C G A-3' and (reverse) 5'-T G C G C A C C T C G G T G T T A A C-3'; Bmi (forward) 5'-T T C C G C C C G C T C A G A T C-3' and (reverse) 5'-C G A T G C A T T T C T G C T T G A T A A A A-3'; Hoxb4 (forward) 5'-G A A G G A A G C A A G A A A A G G A G G A A-3' and (reverse) 5'-G A T T A A A G A T G A A G C C T G G A T C C A T-3'; Gfi1 (forward) 5'-T G C T C C G A G T T C G A G G A C T T-3' and (reverse) 5'-C A G A G A G C G G C A C A G T G A C T T-3'; PU.1 (forward) C C G G A T G T G C T T C C C T T A T C and (reverse) T C T A C C C T C C T C C T C A T C T G A; C/EBP α (forward) G A C C A T T A G C C T T G T G T G T A C T G T A T G and (reverse) T G G A T C G A T T G T G C T T C A A G T T; Egr-1 (forward) C C A T G A A C G C C C A T A T G C T T and (reverse): G C T C A T C C G A G C G A G A A A A G

Methylcellulose assays- 1.5×10^5 splenocytes, 1.5×10^4 bone marrow cells, 500 sorted LSK and CMP and 1000 GMP and MEP were plated in methylcellulose containing IL-3, IL-6, SCF and EPO (M3434, Stem Cell Technologies). In other methylcellose assays, 5×10^5 wild-type or Alox15 bone marrow cells were plated in methylcellulose (M3234, Stem Cell Technologies) after addition of 10 ng/ml M-CSF, G-CSF, GM-CSF or IL-3 (Peprotech). After 10 days, colonies were enumerated using light microscopy in a blinded fashion. In some assays, 1

μM 12(S)-HETE (Cayman Chemical), 10 μM or 50 μM NSC-87877 (EMD bioscience) or 0.025 μM BSO (Sigma) were added to the methylcellulose at the time of plating.

Rapamycin treatment- Rapamycin (LC, laboratories) was dissolved in absolute ethanol into 50 mg/ml stock and then further diluted in 5% Tween-80 (Sigma) and 5% PEG-400 (Sigma). Mice were injected intraperitoneally with 4 mg/kg rapamycin or vehicle control in a 200 μl volume daily for 7 days.

Retroviral Studies- MigR retrovirus was obtained from Dr. W. Pear (University of Pennsylvania). Mutated nondegradable Beta-catenin MSCV was obtained from Dr. T. Reya (Duke University). 12/15-LOX in the MigR retroviral construct was constructed by introducing a Bgl II site using PCR amplification of a plasmid containing 12/15-LOX (C. Funk) followed by subcloning into Topo vector (Invitrogen). The MigR vector and the 12/15-LOX Topo plasmid were digested overnight with Bgl II and XhoI, purified and ligated. Sequence of 12/15-LOX was verified by sequencing (University of Pennsylvania).

To make the retroviral particles, 293T cells were plated at 2×10^6 cells/plate into 6 cm plates in 3 ml. The next day, the media was replaced with warm media containing 25 mM chloroquine. The transfection cocktail was prepared in 1.7ml eppendorf tubes in 500 μl total volume in sterile water: 50 μL 2.5 M CaCl_2 , 5 μg pCL-Eco packaging vector (Imgenex), 10 μg retroviral plasmid. 500 μL HEPES-buffered saline HeBS (2X: 50 mM HEPES, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4 at pH 7.1 filtered and frozen aliquots at -20°C) was added drop-wise with a 1 ml serological pipette and then bubbled and

vortexed for 10 seconds each. The cocktail was slowly added to the side of the dish. After 5-7 hours, cells were washed and medium replaced. Medium was replaced again the following day. At 48 hours post-transfection, viral particles were isolated from the supernatant, filtered through a 0.45 μ M syringe and aliquots stored at -80°C . Titer was checked on 3T3 cells grown at 1×10^6 overnight in 6 cell plates. 100 μ L supernatant and 2 μ g/mL polybrene was added and titer checked for GFP expression by flow cytometry 48-72 hours post-transduction.

For retroviral transduction, B6 and Alox15 E14.5 fetal liver cells were isolated and stimulated with 50 ng/ml SCF, 10 ng/ml IL-3 and IL-6 (Peprotech) at $2-3 \times 10^6$ cells/ml in 4 ml 20% FCS RPMI in 6 well plates. After overnight incubation, 0.5-1 ml was taken, spun and replaced with 0.5-1 ml retroviral supernatant and 4 μ g/ml polybrene (Sigma) was added. Cells were spun for 90 minutes at 2750 rpm and then put in an incubator overnight. The next day, the transduction procedure was repeated. Fetal liver cells were determined to be retrovirally transduced by the expression of GFP in a percentage of the cells at day 3 in culture. Cells were at least 30% GFP⁺. On either day 2 or day 3, cells were washed 3X, counted and at least 1×10^6 cells were injected via tail vein in 200 μ l PBS into lethally irradiated mice (900 rads). For sublethal irradiation, 400-700 rads were used.

Statistical Analysis- One-way ANOVA, t-tests, or Mann-Whitney were applied using Graphpad Prism software or Excel as indicated for each data set. Results in which $p < 0.05$ were considered statistically significant for all tests

CHAPTER 1: Alox15 mice exhibit alterations in hematopoietic compartments

Hematopoiesis is the process whereby all the cells of the immune system develop. It is a tightly regulated process orchestrated by a balance of transcription factor and cytokine signaling that results in defined numbers of blood cell populations including lymphoid, erythroid, and myeloid cell lineages. HSCs are the only cells with both the ability to self-renew and to differentiate into all blood cell types. As cells further differentiate they become more limited in their differentiative capacity. Dysregulated hematopoiesis can result in leukemias, anemias or immunodeficiencies. Therefore, it is important to elucidate pathways that contribute to normal hematopoiesis. Although much is known about transcription factors that regulate cell fate decisions during hematopoietic development, less is known about signaling pathways that govern hematopoietic cell specification.

Although fatty acid metabolism has been extensively studied in the context of chronic inflammation mediated by hematopoietic cells, the role of fatty acid metabolism in general during hematopoietic development is not well understood. In the process of studying the role of 12/15-LOX-mediated fatty acid metabolism in the context of chronic inflammation, our lab discovered that a proportion of Alox15 mice, which lack 12/15-LOX, developed a CML-like disease over the course of a year. Moreover, the majority of Alox15 mice which lacked the CML-like disease were referred to as “asymptomatic” and exhibited an increased percentage of Mac1⁺Gr1⁺ cells in the blood, spleen and bone marrow (BM)

(Middleton et al., 2006b). However, other hematopoietic compartments within Alox15 mice were not studied in depth. Because Alox15 mice exhibited an increased percentage of granulocytes and developed a leukemia, I hypothesized that 12/15-LOX regulates hematopoiesis. To formally test this hypothesis, I characterized the hematopoietic subsets in wild-type and Alox15 mice. This chapter describes the multiple hematopoietic defects in Alox15 mice which demonstrates that 12/15-LOX does indeed regulate hematopoiesis.

Alterations in WBC compartment

To establish the impact of 12/15-LOX deletion on the peripheral hematopoietic compartment, we performed hematologic analysis of peripheral blood from “asymptomatic” 12-15 week old Alox15 and B6 mice. Alox15 mice exhibited a lower white blood cell (WBC) count, largely attributable to a reduction in lymphocytes and monocytes. Although the number of eosinophils and neutrophils were similar, they accounted for a greater percentage of total cells in Alox15 mice. The absolute number of basophils in asymptomatic Alox15 mice was increased as noted previously (Figure 8A; (Middleton et al., 2006b).

To dissect the reduction in lymphocytes in Alox15 mice, we examined mature lymphoid populations in the spleen and lymphoid development in BM and thymus. The reduction in mature lymphocytes was mainly attributed to a reduction in B cells in the spleen (Figure 8B). Moreover, B cell development in the BM was defective in Alox15 mice. Although the percentage of earliest B lineage progenitors, including LSK ($\text{Lin}^- \text{Sca1}^+ \text{cKit}^+$), common lymphoid progenitors (CLP: $\text{Lin}^- \text{IL-7R}\alpha^+ \text{cKit}^{\text{lo}} \text{Sca1}^{\text{lo}}$) and preproB ($\text{B220}^+ \text{CD43}^+ \text{HSA}^-$

CD19⁻) cells were similar between wild-type and Alox15, the percentage of proB (B220⁺CD43⁺HSA⁺CD19⁺) and preB (B220⁺CD43⁻IgM⁺CD19⁺) cells were reduced in Alox15 BM (Figure 8C). We also observed a less dramatic but significant defect in T cell development. Double-positive thymocytes (CD4⁺CD8⁺) were decreased in Alox15 compared to B6, but early thymic progenitors (ETP: Lin⁻CD25⁻cKit⁺), DN2 (Lin⁻CD25⁺cKit⁺) and DN3 (Lin⁻CD25⁺cKit⁻) were similar (Figure 8D).

We next determined whether the defects in the WBC compartment were also defective in younger (6 week), and older (26-30 weeks) mice using hematologic analysis of peripheral blood. Indeed the alterations in WBC, lymphocytes, monocytes and basophils were detected at multiple ages (Figure 9). Moreover, hematologic analysis of the subset of Alox15 mice exhibiting a CML-like MPN, demonstrated increased numbers of WBC compared to asymptomatic Alox15 mice between 26-30 weeks of age. This increase was due primarily to an increase in neutrophils, which confirms the CML-like MPD in these mice (Figure 9).

12/15-LOX regulates RBC development

As 12/15-LOX regulates erythroid development in rabbits and humans, we also investigated whether 12/15-LOX regulates murine erythroid development. Alox15 mice exhibited a decrease in red blood cells (RBC) and a concomitant increase in reticulocyte number at 12-15 weeks of age. Moreover, hemoglobin (HGB) was decreased while mean cell volumes (MCV) of the RBC were increased, indicating that Alox15 mice developed a macrocytic anemia

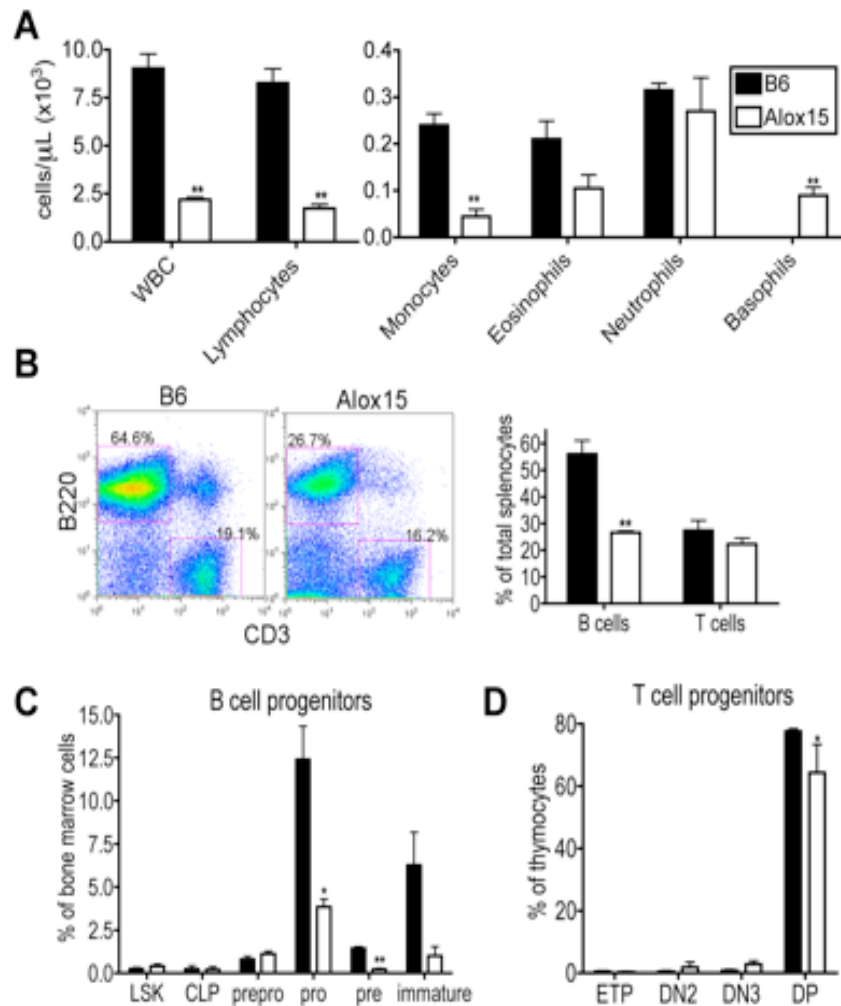


Figure 8. Alox15 mice exhibit multiple hematopoietic defects. (A) Reduction in WBC, lymphocytes, monocytes and increase in basophils in peripheral blood from 12-15 week Alox15 mice compared to B6. (B-C) Defective B cell development in Alox15 mice. (B) Splenocytes were isolated and stained for B cells (B220⁺) and T cells (CD3⁺). Shown is a representative plot and a summary of 5 experiments. (C) BM cells were stained for B cell progenitors and analyzed by flow cytometry: LSK(Lin⁻Sca1⁺cKit⁺), CLP(Lin⁻IL-7R α ⁺cKit^bSca1^b), preproB(B220⁺CD43⁺HSA⁻CD19⁻), proB(B220⁺CD43⁺HSA⁺CD19⁺), preB(B220⁺CD43⁻IgM⁺CD19⁺) and immature B (B220⁺CD43⁻gM⁺CD19⁺). (D) Thymocytes were stained for T cell progenitors and analyzed by flow cytometry: ETP(Lin⁻CD25⁻cKit⁺), DN2(Lin⁻CD25⁺cKit⁺), DN3(Lin⁻CD25⁺cKit⁺) and DP(CD4⁺CD8⁺); *p<.05, **p<.01 compared to wild-type.

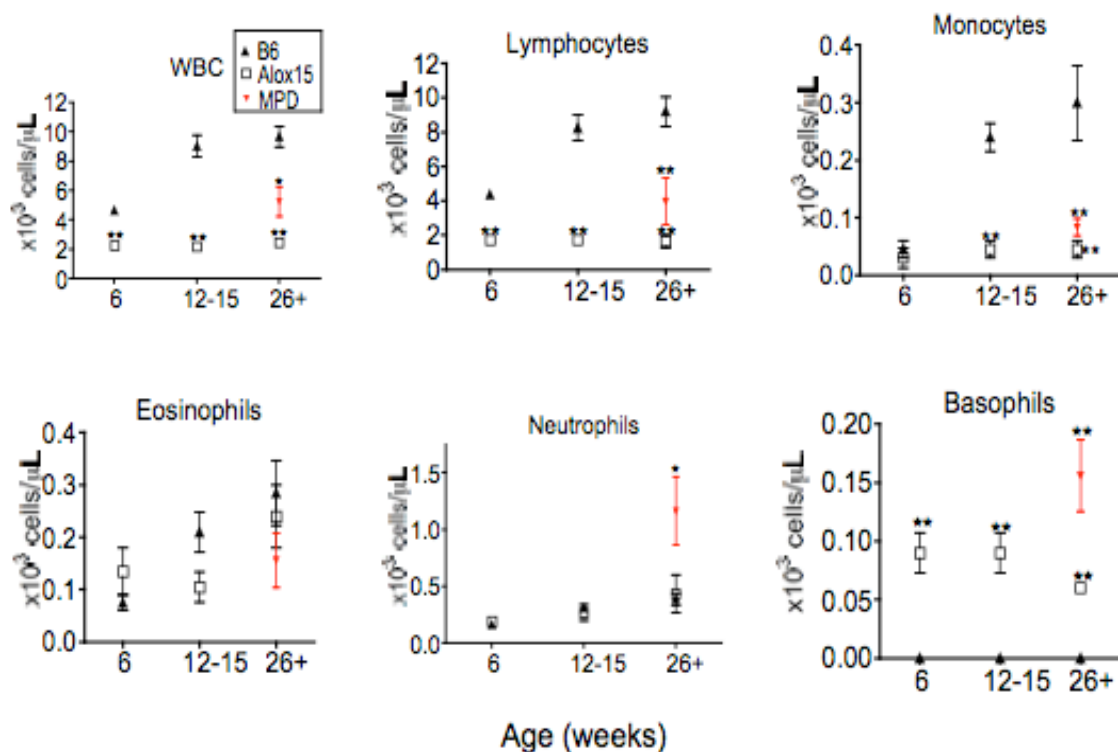


Figure 9. Disrupted hematopoiesis throughout Alox15 lifespan compared to B6. The indicated blood cell populations determined using a cell analyzer demonstrate decreased WBC, lymphocytes, monocytes, and increased basophils in asymptomatic Alox15 mice at 6 weeks, 12-15 weeks, and 26+ weeks of age compared to B6. In Alox15 mice with documented myeloproliferative disease (MPD) there was an increase in the number of neutrophils (n=4). *p<.05, **p<.01 compared to wild-type.

(Figure 10A). ***

Because RBCs were decreased in Alox15 mice, we determined the percentage of erythroid progenitors in the BM and the spleen using Ter119 and CD71 (transferrin receptor) erythroid markers. Despite the decrease in mature RBC, Alox15 erythroid progenitors were expanded, particularly in the spleen, the major site of stress erythropoiesis in mice (Figure 10B). Both proerythrocytes ($CD71^+Ter11^lo$) and erythrocyte progenitors ($Ter119^{hi}$) were expanded. To determine at which point the defect in RBC development occurred, I compared the frequency of B6 and Alox15 cells in erythroblasts fractions A, B and C using CD71 and forward scatter after gating on the $Ter119^+$ progenitors. As erythroblasts develop they exhibit high forward scatter (size) and elevated levels of CD71 (transferrin receptor), which participates in iron transport (Fraction A). As the erythroid progenitors mature, they uptake iron and condense their membranes leading to decreased forward scatter (Fraction B). Eventually the erythroid cells are condensed and lose CD71 expression (Fraction C). Alox15 BM and spleen had increased fraction A, and the Alox15 spleen exhibited an increase in fraction B compared to wild-type. However, there was a comparable percentage of cells in fraction C between wild-type and Alox15 BM and spleen suggesting a block in erythroid differentiation at this stage (Figure 10C). The defect in RBC development likely occurs at the erythroblast B-C transition.

These data are consistent with the reported expansion of splenic red pulp in Alox15 mice (Middleton et al., 2006b). Therefore, I examined H&E stained splenic sections from wild-type and Alox15 mice to identify whether the

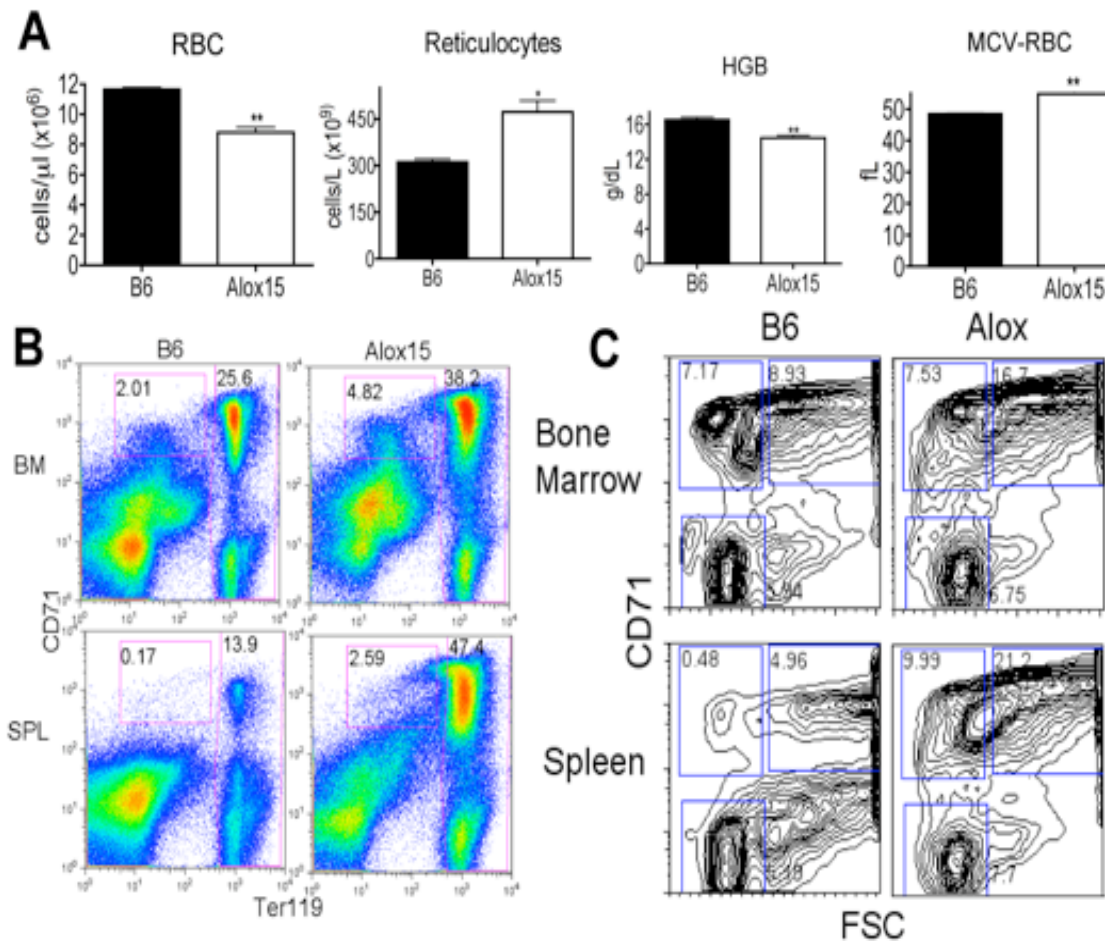


Figure 10. 12/15-LOX regulates erythroid development. (A) Hematologic analysis of peripheral blood from 12-15 week old B6 and Alox15 mice. Numbers of RBC, reticulocytes, hemoglobin (HGB) and mean corpuscular volume of the RBC (MCV-RBC) in B6 and Alox15 is depicted; (B) Stress erythropoiesis in Alox15 mice demonstrated by expansion of erythroid progenitors. BM cells and splenocytes from B6 and Alox15 mice were analyzed by flow cytometry. Proerythrocytes (CD71⁺Ter119^{lo}) and erythrocytes (Ter119^{hi}) are indicated as percentage of total cells for a representative experiment of n=3 in 3 independent experiments. (C) Erythroblast populations are indicated as percentage of total cells. Shown is a representative experiment after gating on Ter119⁺ cells.

expansion of the red pulp was due to the presence of erythroid progenitors. Indeed, Alox15 spleens contained an abundance of erythroid progenitors, which are identified as darkly stained cells, that are relatively absent in wild-type spleens (Figure 11A-B). The presence of erythropoiesis was also demonstrated by megakaryocytes, which were observed in the spleens of Alox15 but not wild-type (Figure 11C). In Alox15 mice with MPN, there was a complete loss of splenic architecture and accumulation of mature and immature granulocytes as previously reported (Figure 11D) (Middleton et al., 2006b).

Alterations in RBC parameters were defined in both young and old mice by hematologic analysis. Both young (6 week) and older (26-30 week) Alox15 mice exhibited decreased RBC and HCT. In addition, older Alox15 mice (26-30 week) exhibited a decrease in platelet count (Figure 12). We also determined the cellular subsets in the blood of the approximately 15% of Alox15 mice over the course of a year that were moribund (Middleton et al., 2006b). This analysis indicated there was a severe decrease in RBC and hematocrit (HCT) as the Alox15 mice progressed from the asymptomatic syndrome to the more aggressive disease (Figure 12).

No significant hematopoietic defects in Alox15 heterozygous mice

We also characterized the hematopoietic compartment of Alox15 heterozygous mice. First we determined that the splenocytes of heterozygous Alox15 mice express roughly half as much 12/15-LOX as wild-type and hence 12/15-LOX is expressed in a gene dose-dependent manner (Figure 13A). However, based on hematological analysis of peripheral blood subsets, there

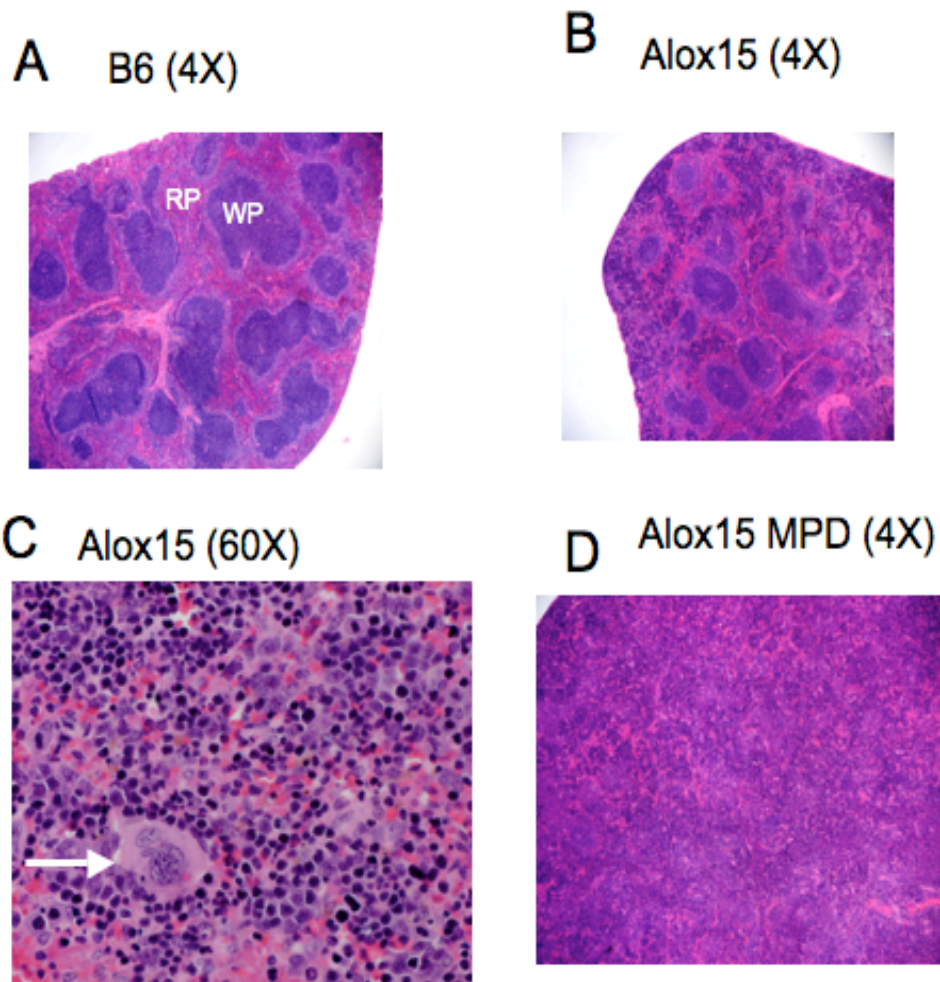


Figure 11. Alox15 exhibits disrupted splenic architecture due to increased numbers of erythroid progenitors. (A) Representative H&E of 12 week B6 splenic section demonstrating separation of white pulp (WP) and red pulp (RP). (B) Representative Alox15 splenic section demonstrating expansion of red pulp by presence of darkly stained cell throughout. (C) 60X of Alox15 splenic section. Shown is a large megakaryocyte (arrow) and darkly stained erythroid progenitors throughout. (D) Loss of splenic architecture in Alox15 spleens with MPD.

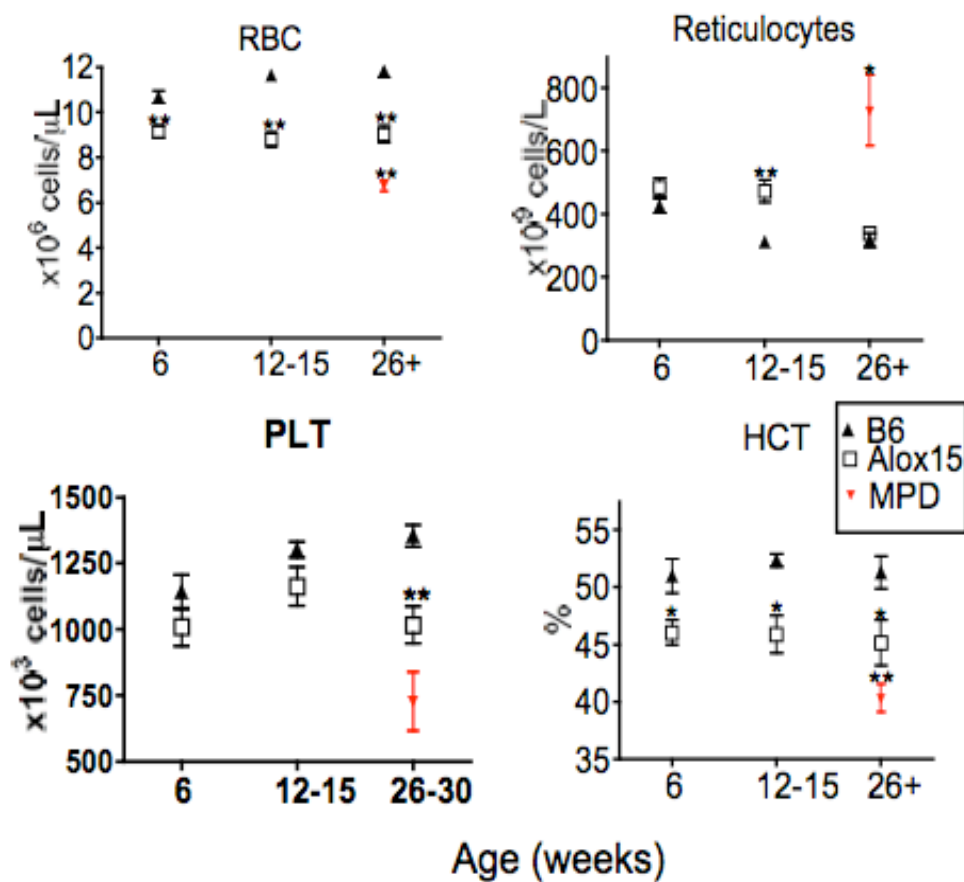


Figure 12. Disrupted erythroid cell parameters throughout Alox15 lifespan compared to B6. The indicated blood cell populations determined using a cell analyzer demonstrate decreased RBC, Platelet (PLT) and HCT in asymptomatic Alox15 mice at 6 weeks, 12-15 weeks and 26+ weeks of age compared to B6. In Alox15 mice with documented myeloproliferative disease (MPD) there was an increase in the number of reticulocytes and a further decrease in RBC and HCT (n=4). *p<.05, **p<.01 compared to wild-type.

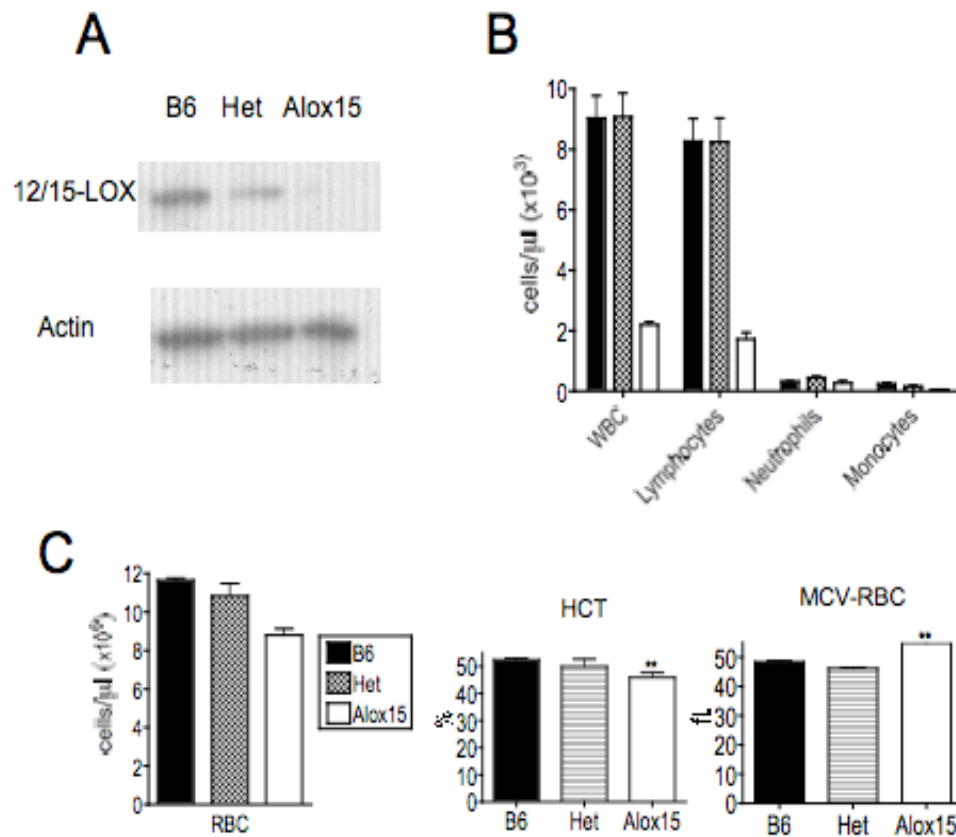


Figure 13. No significant phenotype in heterozygous Alox15

hematopoietic compartment. (A) Immunoblot of B6, Het or Alox15

spleens demonstrating reduced expression of 12/15-LOX in Het spleens.

(B-C) Cell analyzer analysis of (B) WBC and (C) RBC parameters in B6,

Alox15 and Heterozygous mice. n=4 for Het mice.

were no significant defects in the hematopoietic compartment (Figure 13B-C). This may be due to small numbers (n=4) and variability in age of the mice (5-12 weeks). Nonetheless, no significant differences were detected in WBC (Figure 13B) or RBC (Figure 13C) parameters.

Defects in Alox15 mice are cell-autonomous

To determine whether the defects in Alox15 mice were cell-autonomous, we performed adoptive transfer studies to define whether the phenotype was inherent to BM-derived cells and/or dependent on the host environment. B6 or Alox15 donor BM cells were engrafted into lethally irradiated congenic wild-type recipients. At 16 weeks post-engraftment, recipient mice reconstituted with Alox15 cells had decreased total WBC, lymphocytes and RBC in peripheral blood compared to mice reconstituted with B6 BM (Figure 14A), analogous to the defects observed in cells from Alox15 mice. Conversely, when congenic wild-type (B6.SJL) BM was used to reconstitute B6 or Alox15 mice, there were no differences in total or differential white blood counts (Figure 14B). These data indicate that 12/15-LOX regulates hematopoiesis in a cell-autonomous manner.

Implications

Through the comparison of peripheral hematopoietic subsets in wild-type and Alox15 mice, we demonstrate that 12/15-LOX regulates multiple hematopoietic subsets in a cell-autonomous manner. However, although we demonstrate the the phenotype of the blood cell subsets of track with the genotype of the reconstituted bone marrow, it is possible that a subset of bone marrow cells function in a paracrine manner to regulate blood cell

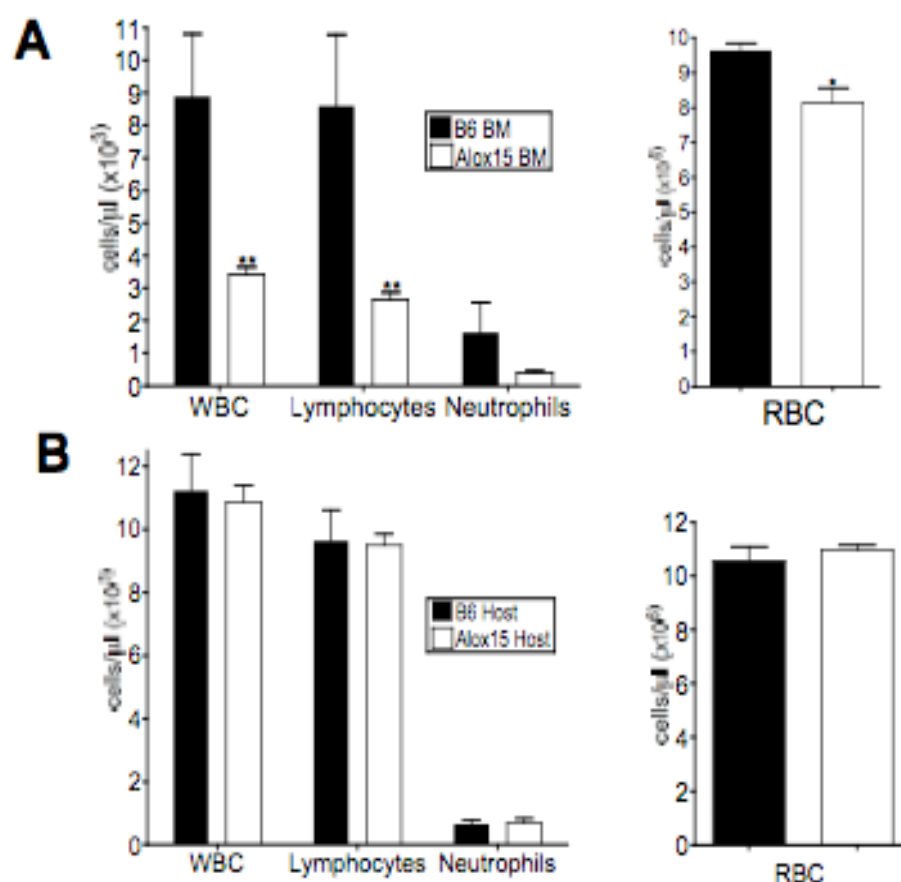


Figure 14. Defects in Alox15 hematopoiesis are cell-autonomous. (A)

Assessment of hematopoietic reconstitution of lethally irradiated B6.SJL mice

16 weeks post-engraftment with 1×10^6 B6 or Alox15 BM cells. (n=6). (B)

Hematopoietic reconstitution of lethally irradiated B6 and Alox15 mice 16

weeks post-engraftment of 1×10^6 B6.SJL BM cells. (n=3); *p<.05, **p<.01

compared to wild-type.

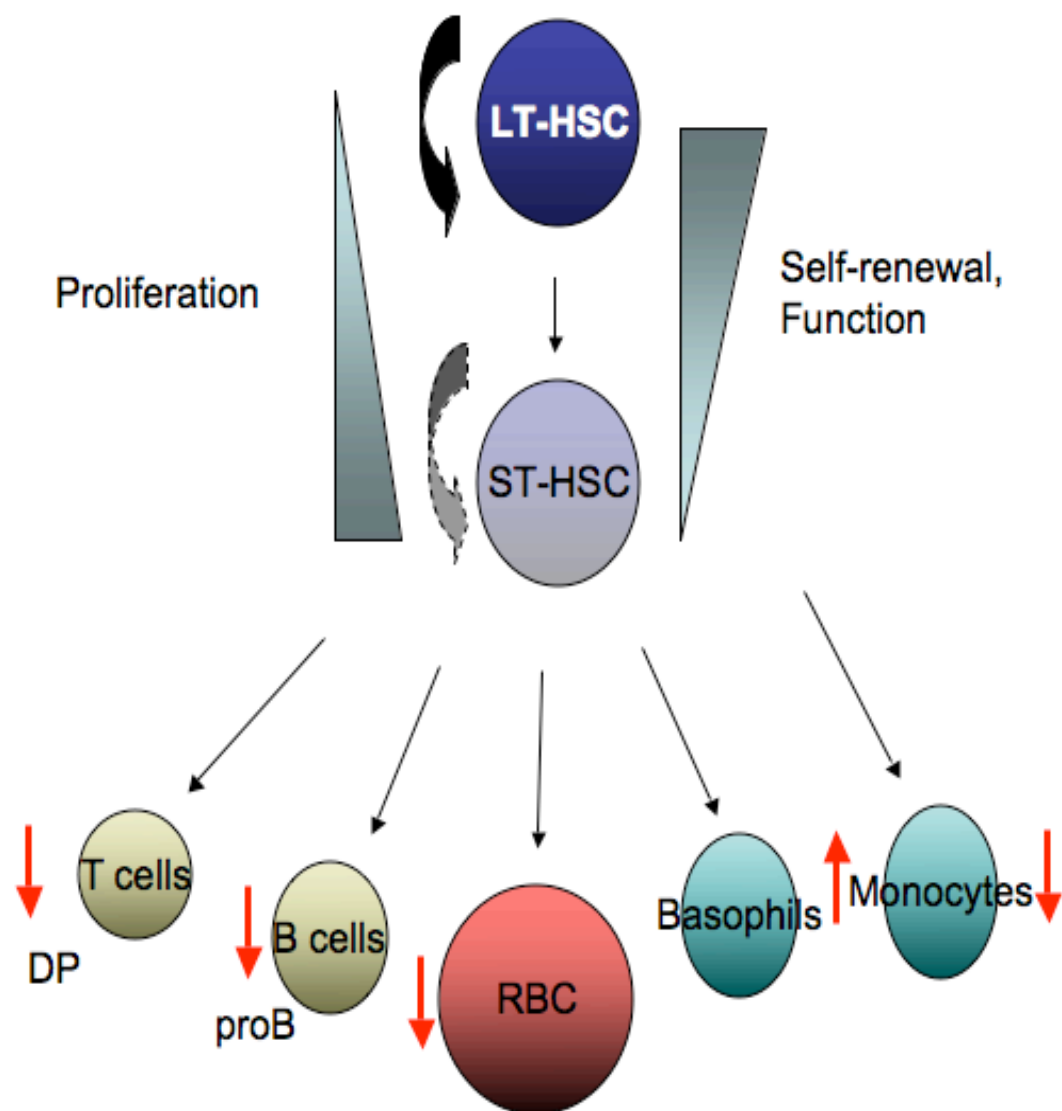


Figure 15. Summary of dysregulated hematopoietic compartment in Alox15

mice. Alox15 mice exhibit decreased DP T cells, decreased B cells due to a block at proB cell stage, macrocytic anemia, increased basophils and decreased monocytes.

development. We more definitely demonstrate that the defect in blood cell development is cell-intrinsic hematopoietic stem cells using competitive reconstitution assays in Chapter 2 of this dissertation. 12/15-LOX regulates T cell differentiation at the DP stage, B cell differentiation at the pro-preB cell stage, RBC generation, monocyte number and limits the number of basophils (Figure 15). The presence of multiple hematopoietic defects in the Alox15 mice suggests that 12/15-LOX may regulate a common progenitor such as the hematopoietic stem cell. The role of 12/15-LOX in hematopoietic stem cell function will be discussed in Chapter 2.

Although these data suggest that 12/15-LOX may regulate a common progenitor such as the HSC, it is also possible that 12/15-LOX may independently directly regulate multiple hematopoietic subsets. These possibilities are not mutually exclusive and our data as well as previous findings suggest that both these possibilities do in fact simultaneously occur. Our data imply that 12/15-LOX may possibly regulate development of B and T lymphocytes, erythrocytes, and monocytes by distinct mechanisms by which it regulates HSC function.

The mechanism whereby 12/15-LOX regulates B and T cell development remains to be elucidated. Interestingly, 12/15-LOX had not been previously shown to regulate lymphoid cell populations though its products were found in the thymus (Harizi et al., 2008). Interestingly, IRF-8, which we demonstrated is regulated by 12/15-LOX, had been shown to regulate B cell development at the same stage of development that is defective in Alox15 mice (pro-preB cell

stage) (Lu et al., 2003). However, whether 12/15-LOX regulates B cell differentiation through IRF-8 is currently unknown and is a potentially interesting future direction.

The defect in late stage erythroid differentiation in Alox15 mice is in concordance with previous observations that a functional homolog of 12/15-LOX, 15-LOX, and its specific products, 15(S)-HETE and 13(S)-HODE, in other species regulate reticulocyte maturation by modulating membrane degradation (Kuhn and Brash, 1990; van Leyen et al., 1998). 12/15-LOX had not been previously shown to regulate RBC development in mice and this is the first study that establishes its role in murine erythroid cell differentiation. To more fully understand the development of the macrocytic anemia in Alox15 mice, it would be an interesting future direction to elucidate the mechanism whereby 12/15-LOX regulates murine reticulocyte maturation.

There were decreased blood monocytes in Alox15 mice suggesting that 12/15-LOX may regulate monocyte development and myelopoiesis. The role of 12/15-LOX in myeloid cell development is of interest because of the potential implications for CML and will be addressed further in Chapter 3 of this thesis.

CHAPTER 2: 12/15-LOX is a critical regulator of LT-HSC self-renewal

The presence of multiple hematopoietic defects in Alox15 mice, detailed in Chapter 1, suggests two possibilities, either of which are plausible and not necessarily mutually exclusive. The first possibility is that 12/15-LOX regulates multiple stages of hematopoiesis independently. The second possibility is that 12/15-LOX regulates a common hematopoietic progenitor such as HSC. The data presented in this and subsequent chapters demonstrate that both these possibilities occur. Specifically, I will present data in Chapter 3 which demonstrate that 12/15-LOX drives myelopoiesis by promoting ICSBP/IRF-8 nuclear accumulation through redox signaling. In this chapter, I will present data which demonstrate that 12/15-LOX regulates HSC function and self-renewal in associated with modulation of canonical Wnt signaling.

The signaling pathways that govern the complex processes of HSC function are not completely understood. HSCs are relatively rare yet must produce billions of new cells daily and be able to adjust their output to respond to environmental stresses such as infection, chemotherapy, blood loss, injury, etc. To accomplish this, HSCs must divide asymmetrically by balancing self-renewal and differentiation. Skewing of asymmetric division results in defective HSC function. Decreases in numbers, alterations in the balance of asymmetric division and increased proliferation decreases the ability of HSC to function (Ficara et al., 2008; Nemeth et al., 2006; Passegue et al., 2005). Although much is known about transcription factors that regulate these processes, such as Gfi-1, Bmi-1, Hox4b and Pu.1 (Hock et al., 2004; Iwama et al., 2004; Iwasaki et al., 2005;

Sauvageau et al., 1995), little is known about the signaling pathways that regulate HSC function. Canonical Wnt signaling is one known pathway that regulates HSC function by maintaining quiescence and initiating a self-renewal program of gene transcription (Fleming et al., 2008; Zhao et al., 2007; Luis et al., 2009). In this chapter, I determine that 12/15-LOX is a novel regulator of canonical Wnt signaling which in turn may regulate HSC function by maintaining quiescence and self-renewal.

Products generated by 12/15-LOX have been implicated in hematopoiesis. ROS, which are generated as a product of 12/15-LOX fatty acid metabolism, can impair HSC function by ROS-dependent regulation of the p38 MAPK pathway (Ito et al., 2006). Moreover, 12/15-LOX generated lipid products, 12(S)-HETE and 15(S)-HETE, can inhibit differentiation of human CD34⁺ hematopoietic progenitors in methylcellulose assays (Desplat et al., 2000). However, a role for 12/15-LOX per se has not been previously established in hematopoietic development and HSC function.

In this chapter, I define 12/15-LOX as a critical regulator of LT-HSCs. 12/15-LOX regulates HSCs by promoting quiescence and self-renewal. Defective HSC function in Alox15 mice is associated with defects in select products of 12/15-LOX including 12/15-LOX-generated ROS, 12(S)-HETE and 13(S)-HODE. 12/15-LOX regulates nuclear ICSBP/IRF-8 accumulation in hematopoietic progenitors, which results in altered expression of ICSBP/IRF-8 target genes, including increased production of Dab2, a negative regulator of canonical Wnt signaling. Consequently, canonical Wnt signaling measured by its

downstream mediator, β -catenin, is disrupted in Alox15 progenitors. Thus, we have defined 12/15-LOX as a novel regulator of LT-HSC function and canonical Wnt signaling.

Alox15 mice exhibit a primary defect in HSC function

To test the possibility that 12/15-LOX regulates HSC function, we needed to determine expression of 12/15-LOX in HSC. Using real time PCR analysis, we determined that 12/15-LOX mRNA was expressed in wild-type HSC isolated by cell sorting (Figure 16A). Moreover, 12/15-LOX protein was expressed in B6 (but not Alox15) Lin⁻cKit⁺ hematopoietic progenitors by immunoblot (Figure 16B).

HSC function can be assessed by the ability to reconstitute the hematopoietic compartment of a lethally irradiated animal. Competitive reconstitution experiments, in which BM from control and experimental mice are mixed with wild-type BM and injected into lethally irradiated mice, are based on two assumptions: that HSC numbers and homing of the HSCs are similar between control and experimental BM cells. LSK were present in similar numbers in B6 and Alox15 BM (Figure 16C). Moreover, B6 and Alox15 BM cells homed with comparable efficiency to both normal and lethally irradiated recipient BM (Figure 16D). It was therefore appropriate to proceed with competitive reconstitution assays.

To elucidate whether 12/15-LOX regulates early hematopoietic progenitors such as the HSC, we set up competitive reconstitutions in which B6 or Alox15 BM cells (CD45.2) were mixed 9:1 with wild-type competitor B6.SJL BM cells (CD45.1) and engrafted into lethally irradiated B6.SJL recipient

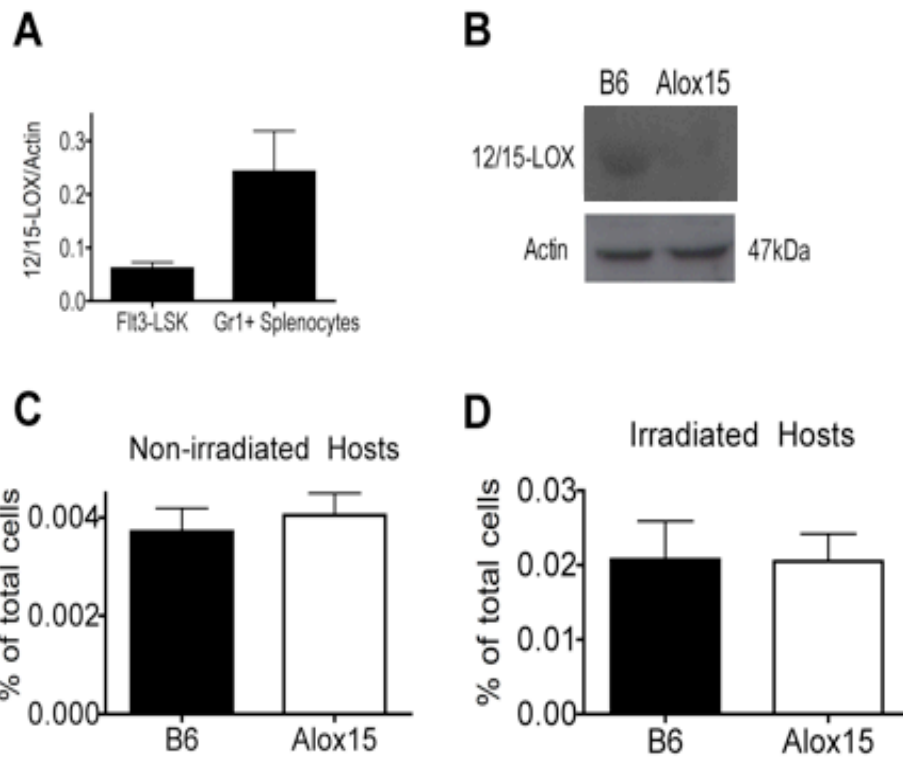


Figure 16. 12/15-Lox is expressed in HSC and does not effect homing to BM. (A) Q-PCR of 12/15-Lox mRNA levels of the indicated populations obtained by cell sorting demonstrate that 12/15-Lox is expressed in HSCs (Lin-Sca1⁺cKit⁺ Flt3⁺) (n=2). (B) 12/15-Lox protein expression in progenitors. 12/15-Lox expression was determined on column-purified B6 and Alox15 Lin⁻cKit⁺ cells by immunoblot on whole protein lysates. (C-D) Similar homing of C57BL/6 and Alox15 bone marrow cells to bone marrow. C57BL/6 and Alox15 bone marrow cells were labeled with PKH67 or PKH26 (Sigma) as per manufacturer's instructions. Cells were then mixed in a 1:1 ratio and 4x10⁶ cells total were i.v. injected into (C) non-irradiated wild-type recipient mice or (D) recipient mice that were irradiated with 1000 rads. After 24 hours, the percentages of C57BL/6 or Alox15 bone marrow cells in the recipient bone marrow were assessed using flow cytometric analysis for PKH67 and PKH26 (n=3) p>.05

mice. Reconstitutions were monitored by bleeding the recipient mice every four weeks and analyzing the frequency of CD45.2 cells, which were derived from B6 or Alox15 BM cells, amongst the total CD45⁺ population (Figure 17). At all time-points post-reconstitution, the contribution of Alox15-derived total CD45⁺ cells, T cells, B cells and granulocytes were dramatically reduced compared to the contribution of B6 donor-derived cells (Figure 18A). After 16 weeks, BM was isolated and analyzed for progenitor subsets. Alox15 HSC were at a profound disadvantage compared to B6-derived HSC under competitive conditions as evidenced by a marked reduction in the proportion of Alox15-derived HSC compared to B6-derived HSC in recipient mice (Figure 18B). In addition to the HSC compartment, we also examined the contribution of B6 and Alox15 cells to B, T and myeloid cell development by gating on CD45.2. In addition to the defect in HSC, Alox15-derived cells exhibited additional defects at later stages of differentiation similar to those described in Chapter 1 (Figure 19). There was a further bias toward competitor-derived cells within B cell and T cell compartments compared to Alox15-derived HSC (Figure 19A-B). Moreover, the defect in more mature Alox15-derived cells was evident as early as four weeks following reconstitution in the blood, when cells were derived from more differentiated progenitors rather than the HSC (Figure 18A).

Additional competitive reconstitution assays using B6 and Alox15 BM mixed 1:1 with congenic competitor cells demonstrated that limiting numbers of Alox15 BM resulted in a further decrease in Alox15-derived HSC during competitive reconstitution assays (Figure 20).

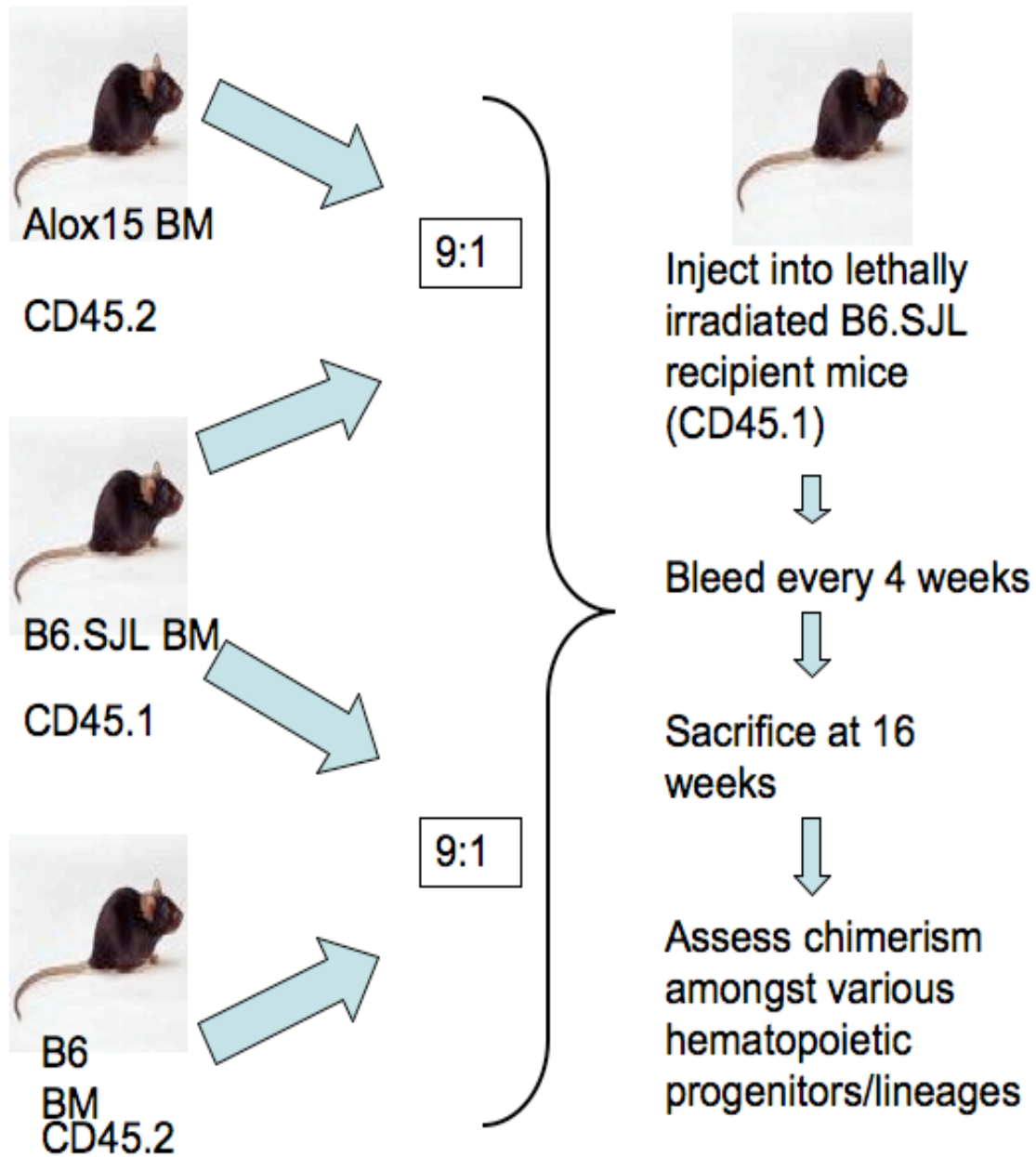


Figure 17. Schematic of competitive reconstitution assays. BM cells from B6 or Alox15 mice (CD45.2) were mixed 9:1 with congenic wild-type bone marrow (CD45.1) and injected into lethally irradiated mice. The reconstitution was monitored by bleeding the recipient mice every four weeks. At 16 weeks the recipient mice were sacrificed and the chimerism was assessed amongst the progenitor populations.

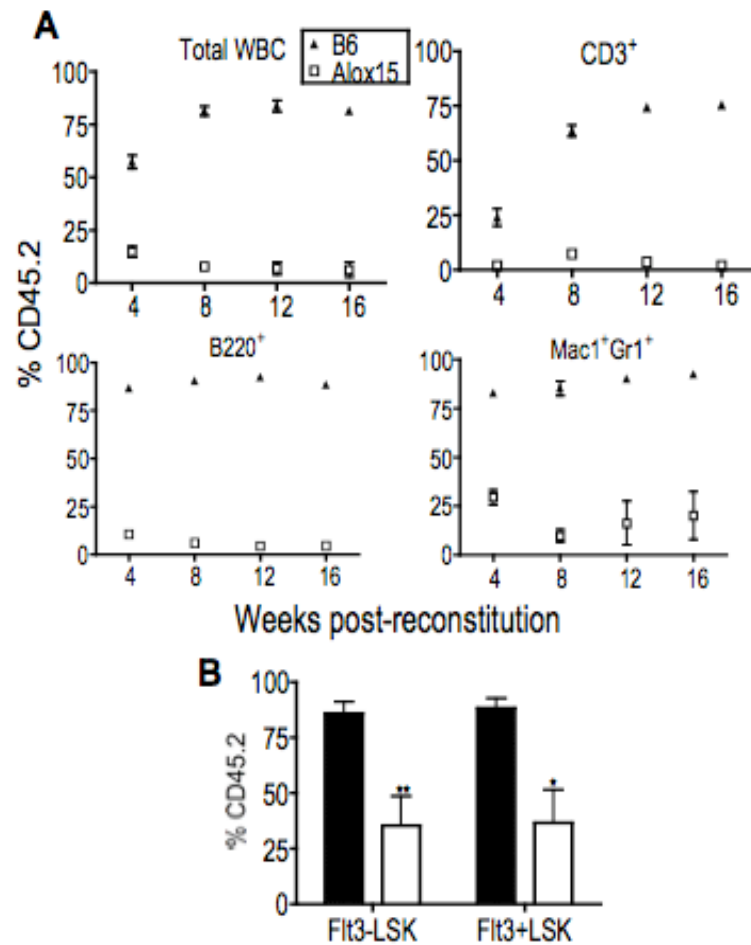


Figure 18. Alox15 HSCs are functionally defective. (A) Lethally irradiated congenic B6.SJL mice were reconstituted with a 9:1 ratio of B6 (solid triangles) or Alox15 (open squares) (CD45.2, test) BM cells to B6.SJL (CD45.1, competitor) BM cells. Reconstitution was monitored by quantifying the percentage of donor-derived CD45.2⁺ blood cells in recipients using flow cytometry at the indicated times. The percentages of CD45.2⁺ total leukocytes (CD45⁺ cells), CD3⁺ cells, B220⁺ cells, and Mac1⁺Gr1⁺ are depicted (n=6); p<.01. (B) Reduction in Alox15 LT-HSCs following during competitive reconstitution. 16 weeks post-engraftment, the percentages of B6 and Alox15 CD45.2 donor-derived Flt3-LSK and Flt3⁺LSK in 9:1 competitively reconstituted mice were determined by flow cytometry (n=6); *p<.05, **p<.01.

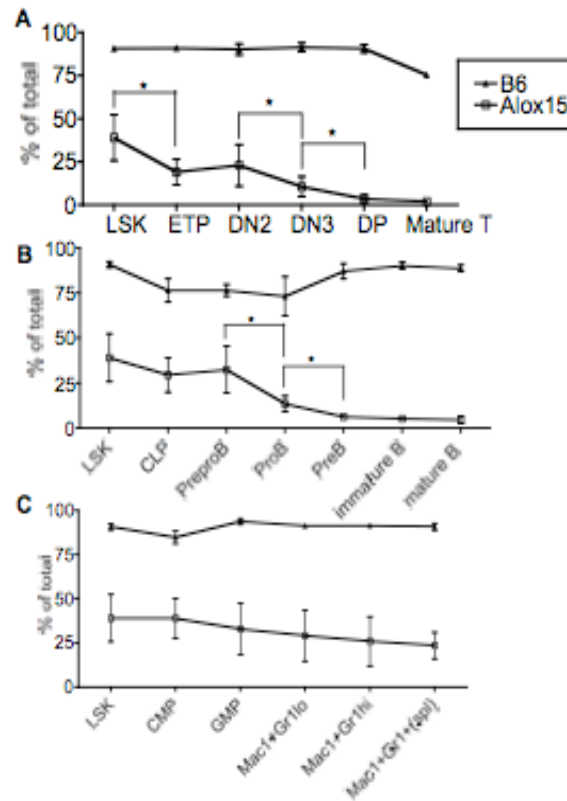


Figure 19. Multiple defects in Alox15 progenitor development during 9:1 competitive reconstitution. After 16 weeks post-reconstitution, the contribution of C57BL/6 or Alox15-derived cells to progenitor development was quantified by determining the percentage of CD45.2⁺ cells at the indicated developmental stage compared to LSK (Lin⁻ Sca1⁺ cKit⁺) cells (A) T cell progenitors in the thymus: ETP (Lin⁻ CD25⁻ cKit⁺), DN2 (Lin⁻ CD25⁺ cKit⁺), DN3 (Lin⁻ CD25⁺ cKit⁺), DP (CD4⁺ CD8⁺) and in the blood mature T cells (CD3⁺); (B) B cell development in the bone marrow: CLP (Lin⁻ IL-7R α ⁺ cKit^b Sca1^b), preproB (B220⁺ CD43⁺ HSA⁻ CD19⁻), proB (B220⁺ CD43⁺ HSA⁺ CD19⁺), preB (B220⁺ CD43⁻ IgM⁻ CD19⁺), immature B (B220⁺ CD43⁻ IgM⁺ CD19⁺), and in the blood mature B cells (B220⁺); (C) myeloid cell progenitors in the bone marrow CMP (Lin⁻ Sca1⁻ cKit⁺ CD16/32^b CD34^{hi}) GMP (Lin⁻ Sca1⁻ cKit⁺ CD16/32^{hi} CD34^{hi}), MEP (Lin⁻ Sca1⁻ cKit⁺ CD16/32^b CD34^b) Mac1⁺Gr1^b, Mac1⁺Gr1^{hi} and in the spleen Mac1⁺Gr1⁺ cells (n=6). *p<.05 compared to the indicated progenitor stage

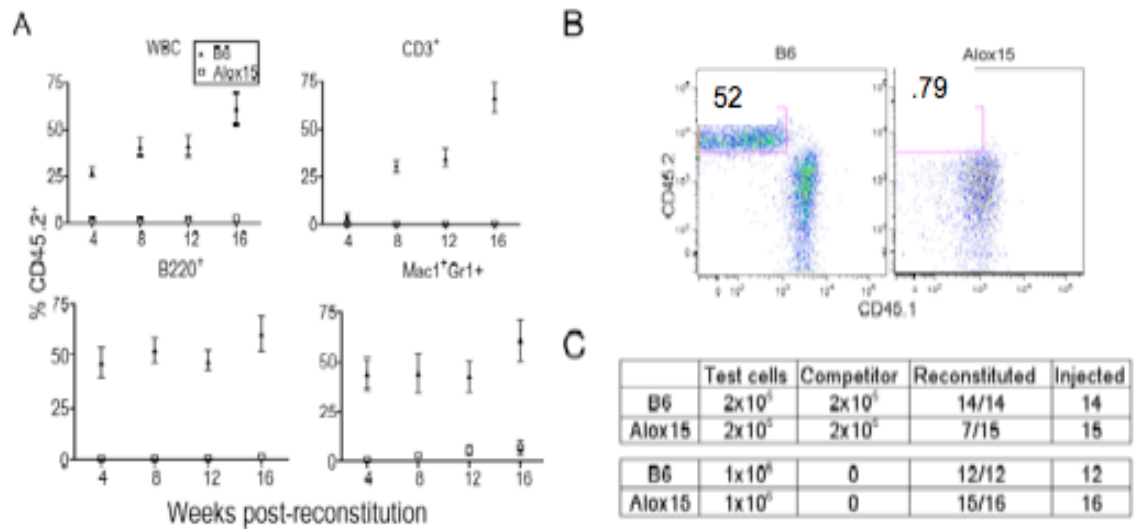


Figure 20. Alox15 hematopoietic development is defective during 1:1 competitive reconstitutions. (A) The percentage of cells derived from B6 and Alox15 bone marrow cells was determined by isolating blood from recipient mice every four weeks and quantifying CD45.2⁺ cells using flow cytometric analysis from the indicated subsets. (B) Representative flow cytometric analysis of CD45.2 cells gated on LSK cells 16 weeks post 1:1 reconstitution. (C) Summary of 1:1 competitive reconstitutions and adoptive transfer experiments. C57BL/6 or Alox15-derived bone marrow was considered to contribute to reconstitution if the percentage of CD45.2⁺ cells in the LSK compartment was greater than 2%.

We also performed competitive reconstitution assays with Alox15 heterozygous cells. Similar to the lack of blood cell defects in Alox15 heterozygous mice (Chapter 1), HSCs from Alox15 heterozygous mice were not defective during competitive reconstitution assays compared to wild-type (Figure 21). Importantly, however, Alox15 HSCs were defective compared to Alox15 heterozygous HSC (Figure 21).

Defect in Alox15 HSC is primary

Multiple hematopoietic defects are present in Alox15 mice as described in Chapter 1, including lymphocytes, monocytes and RBCs. Rather than a primary defect in HSCs, it is possible that the reduced function of Alox15 HSCs is caused by their need to continually replenish the more mature hematopoietic compartments. Nonetheless, as the competitor cells provided a virtually normal hematopoietic compartment during competitive reconstitution assays, these data indicate that the defect in Alox15 HSCs was primary rather than secondary to abnormalities in hematopoietic cells at later stages of differentiation. However, to formally exclude the possibility that the defect in Alox15 HSCs was secondary to later hematopoietic defects, we repeated the competitive reconstitution assays with E14.5 fetal liver cells. Importantly, we found that Alox15 E14.5 fetal livers were similar to B6 based on H&E staining and by flow cytometric analysis of hematopoietic subsets and exhibited no overt phenotype (Figure 22A-B). In the competitive reconstitution assays, B6 and Alox15 fetal liver cells were mixed 2:1 with normal congenic bone marrow to ensure readout of fetal liver-derived HSC function in the context of a normal hematopoietic compartment. Even under

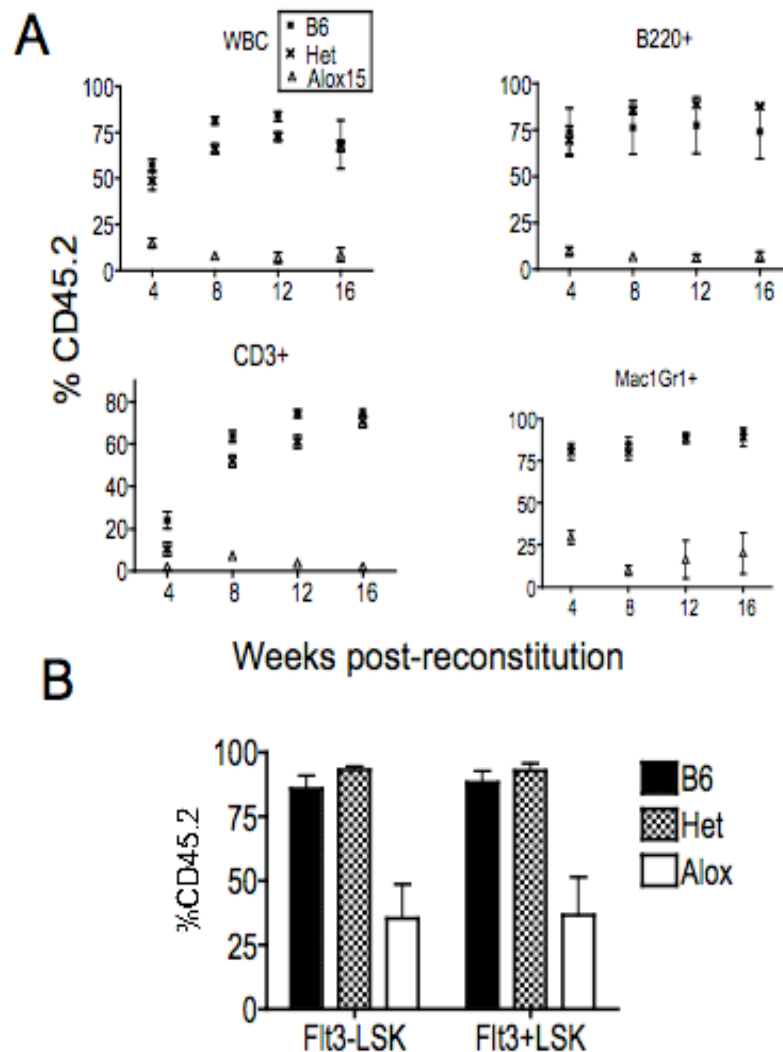


Figure 21. Alox15 heterozygous mice do not display gross defects in HSC function. (A) HSC function was assessed by mixing C57BL/6, Alox15 heterozygous mice or Alox15 homozygous bone marrow (CD45.2) 9:1 with congenic wild-type bone marrow (CD45.1) and injecting into lethally irradiated mice. Reconstitution was monitored by analyzing blood cell CD45.2 populations by flow cytometry. (B) 16 weeks post-engraftment, the percentages of B6, heterozygous and Alox15 CD45.2 donor-derived Flt3-LSK and Flt3+LSK in 9:1 competitively reconstituted mice were determined by flow cytometry

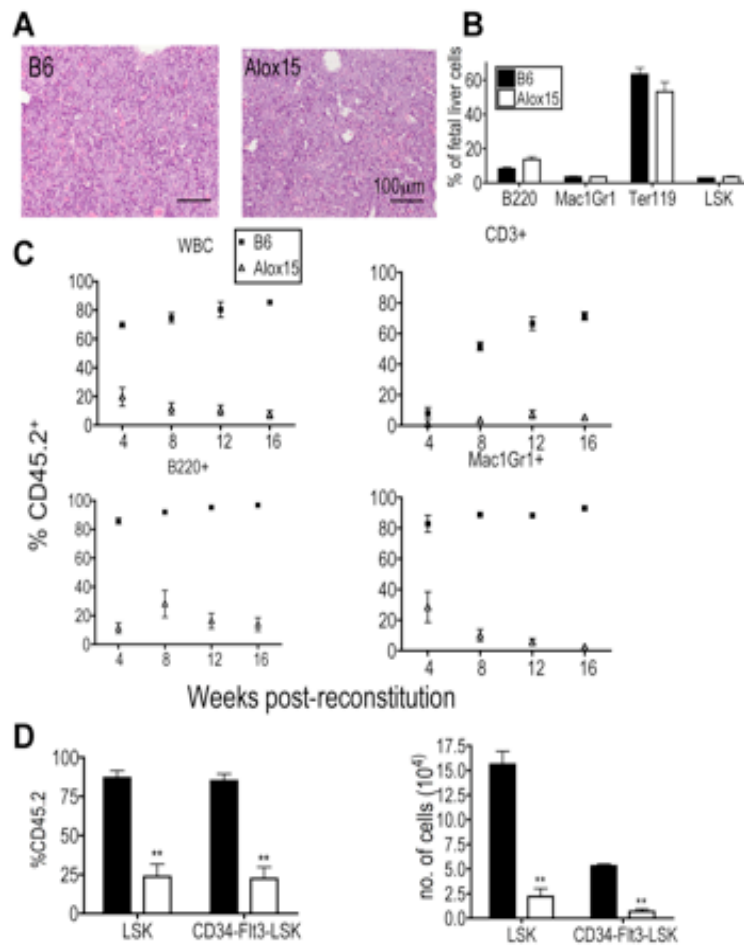


Figure 22. 12/15-LOX directly regulates HSC function. (A) Morphology and (B) cellularity of Alox15 E14.5 fetal livers are normal. (A) Representative H&E staining of B6 and Alox15 E14.5 fetal livers (20X). (B) Hematopoietic subsets in B6 and Alox15 E14.5 fetal livers analyzed by flow cytometry. (C-D) HSC-derived from Alox15 fetal livers are functionally defective. Analysis of hematopoietic reconstitution of lethally irradiated congenic mice at indicated times post-engraftment of B6 or Alox15 E14.5 fetal liver cells mixed 2:1 with congenic competitor BM cells. (C) CD45.2⁺ white blood cell subsets were determined by flow cytometry. (D) Indicated progenitor populations in BM harvested 16 weeks post-transfer were analyzed for CD45.2⁺ cells (n=5). *p<.05, **p<.01.

these conditions, the contribution of Alox15-derived cells to the blood cell populations was significantly reduced compared to B6 at all times post-transfer into lethally irradiated congenic recipient mice (Figure 22C). Moreover, Alox15-derived LSK and CD34⁻Flt3⁻LSK cells in the BM were significantly reduced compared to wild-type (Figure 22D). These experiments were also conducted with fetal liver cells mixed 1:1 with wild-type congenic competitive BM cells with similar results (Figure 23). These data effectively demonstrate that the defect in Alox15 HSCs is not secondary to defects in mature hematopoietic subsets. Rather, 12/15-LOX directly regulates HSC function.

12/15-LOX regulates numbers, proliferation and function of LT-HSC

We further compared the distribution of HSCs within the LSK population of B6 and Alox15 mice (Wilson et al., 2007; Wilson et al., 2008). Interestingly, Alox15 BM had a decreased percentage of primitive dormant LT-HSC (CD34⁻CD150⁺CD48⁻Flt3⁻LSK) and an increased percentage of more differentiated MPP1 (CD34⁺CD150⁺CD48⁺Flt3⁻LSK) compared to B6 (Figure 24A). The LT-HSC population is highly enriched for HSC function and quiescent cells while the more differentiated cells are highly proliferative and have decreased capacity to self-renew. These data suggest that 12/15-LOX regulates the most primitive LT-HSCs.

To determine whether the defect in Alox15 HSCs in the BM competitive reconstitution assays was due to 12/15-LOX regulation of the absolute number of LT-HSCs or the function of LT-HSCs, we performed competitive reconstitution assays using equal numbers of purified LT-HSCs. Five hundred CD34⁻Flt3⁻

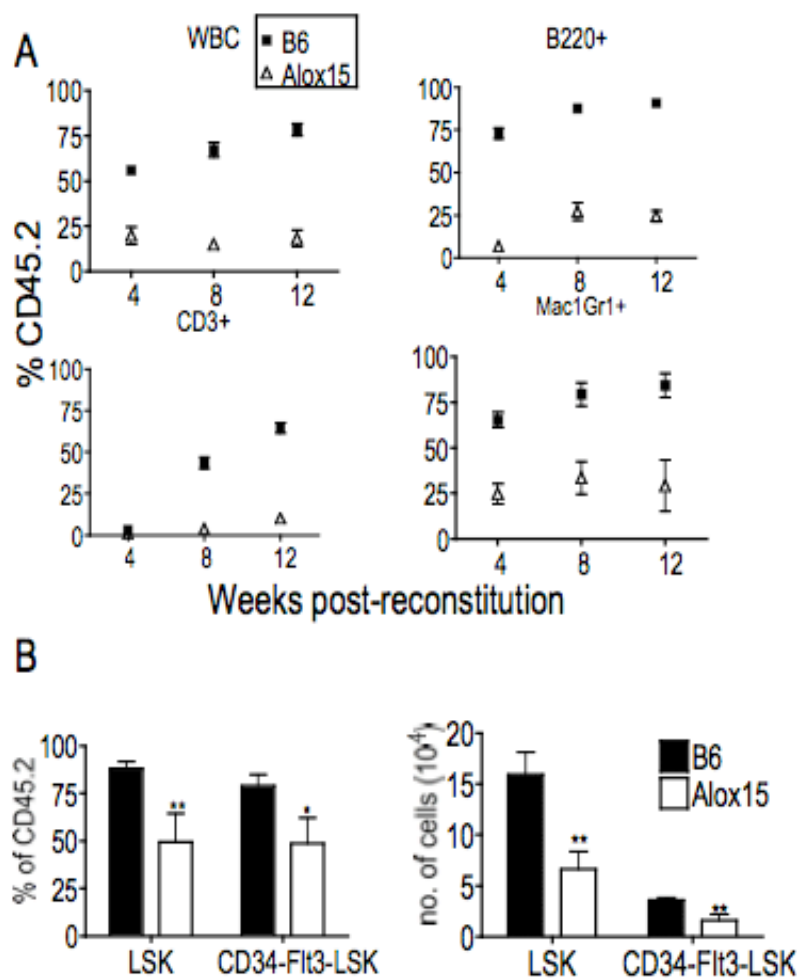


Figure 23. HSC-derived from Alox15 fetal livers are functionally defective during 1:1 competitive reconstitutions. Analysis of hematopoietic reconstitution of lethally irradiated congenic mice at indicated times post-engraftment of B6 or Alox15 E14.5 fetal liver cells mixed 1:1 with congenic competitor BM cells. (A) CD45.2⁺ white blood cell subsets were determined by flow cytometry. (B) Indicated progenitor populations in BM harvested 12 weeks post-transfer were analyzed for CD45.2⁺ cells (n=5). *p<.05, **p<.01.

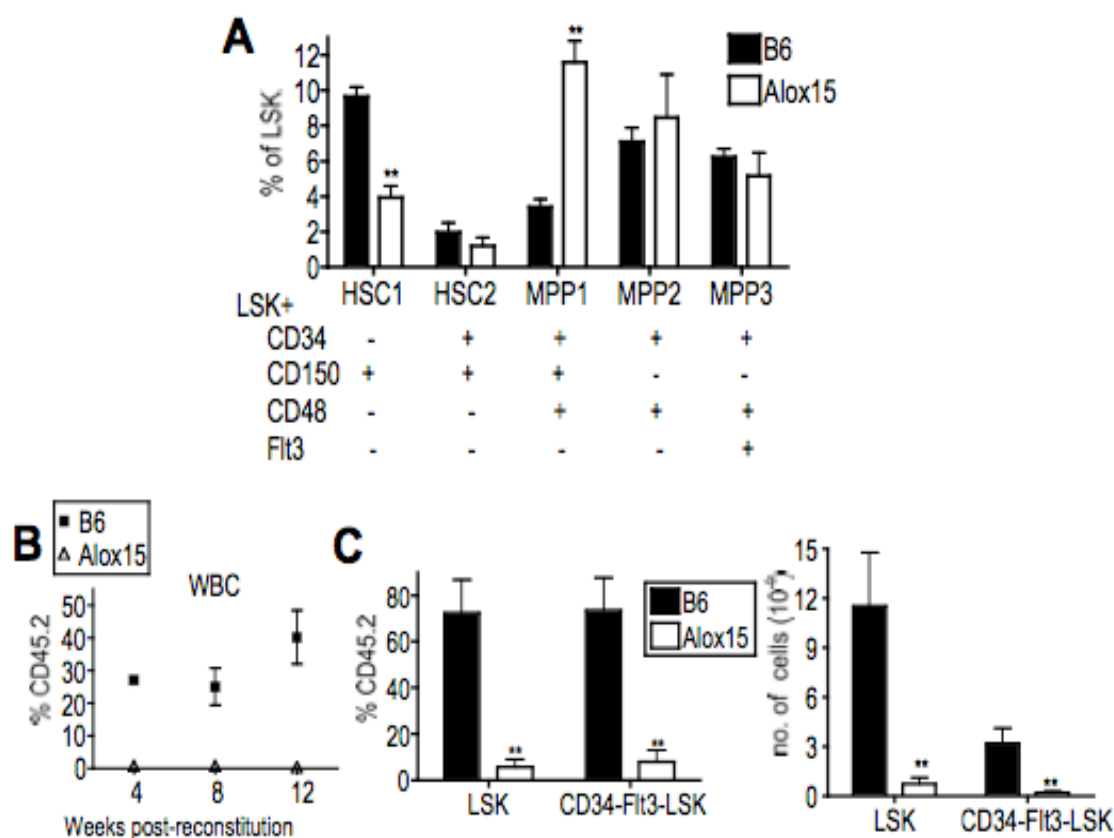


Figure 24. 12/15-LOX regulates the function of LT-HSCs. (A) Flow cytometric analysis of phenotypic subsets demonstrated depletion of the most primitive HSC phenotype and a concomitant increase in a more mature MPP phenotype in Alox15 compared to B6 LSK (n=5); (B-C) 12/15-LOX regulates the function of CD34⁺LSK. Lethally irradiated congenic mice were reconstituted with 500 CD34⁺Flt3⁺LSK cells from B6 or Alox15 BM mixed with 2x10⁵ competitor cells. (B) Reconstitution analyzed by flow cytometry of CD45.2⁺ of total CD45⁺ white blood cells. (C) 12 weeks post-transfer BM was analyzed for CD45.2⁺ LSK and CD34⁺Flt3⁺ LSK by flow cytometric analysis (n=7)

LSK cells, purified by cell sorting from B6 or Alox15 BM, were mixed with wild-type congenic competitor BM cells and injected into lethally irradiated recipient mice. At all times post-reconstitution, there was a significant decrease in the percentage of Alox15-derived blood cells (Figure 24B). Analysis of BM at 12 weeks post-reconstitution showed a decrease in the percentage and absolute number of Alox15-derived LSK and CD34⁺Flt3⁺LSK compared to B6 (Figure 24C). These data demonstrate that 12/15-LOX regulates the function of LT-HSC.

To determine the mechanism whereby 12/15-LOX regulates LT-HSC, we compared the rates of cell death and proliferation in Alox15 and B6 HSC. B6 and Alox15 LSK exhibited comparable levels of cell death *ex vivo* using both TUNEL and AnnexinV/DAPI staining (Figure 25A-B). In addition, B6 and Alox15 LSK exhibited similar cell death after sorting and *in vitro* culture with limiting levels of cytokines (Figure 25C). Thus, 12/15-LOX does not regulate apoptosis of HSC. However, Alox15 LSK contained an increased percentage of cycling cells compared to wild-type (Figure 26A). This difference was most dramatic in the CD34⁺LSK subset in which there was a decrease in the percentage of Alox15 cells in G₀ (31.9% vs. 59.7%) but increased percentage of cells in G₁ (38.5% vs. 17.3%) and S/G₂M (24.0% vs. 11.6%) (Figure 26B). Thus, 12/15-LOX regulates the number, proliferation and function of primitive LT-HSCs. As LT-HSCs proliferate, they may differentiate rather than self-renew leading to depletion of the LT-HSC population. To formally test this hypothesis, we next tested whether 12/15-LOX regulates self-renewal.

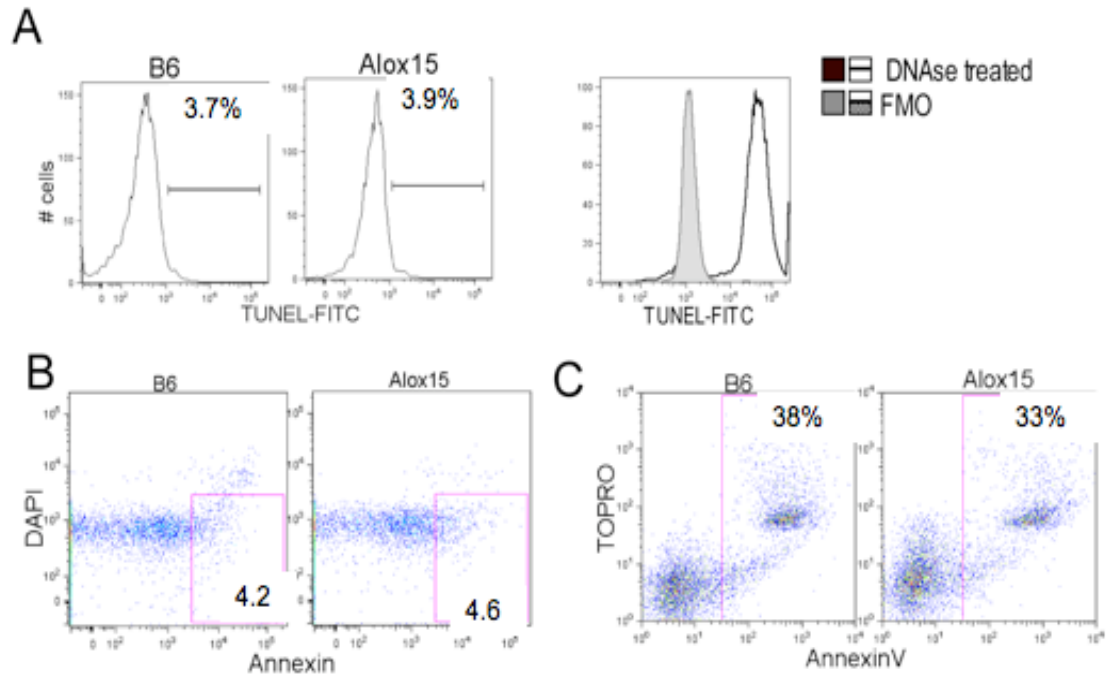


Figure 25. 12/15-LOX does not regulate apoptosis of LSK. (A-B) B6 and Alox15 LSK display similar levels of apoptosis *ex vivo* determined by (A) TUNEL and (B) AnnexinV/DAPI staining. A representative flow cytometric analysis gated on LSK cells is depicted and a negative control Fluorescence Minus One (FMO-shaded) and positive DNase control (open) is shown for TUNEL staining (n=3); p>.05 (C) B6 and Alox15 LSK display similar levels of apoptosis *in vitro*. B6 and Alox15 LSK were isolated by cell sorting and cultured for 48 hours in the presence of 25ng/ml SCF. Apoptosis was determined by flow cytometric staining using AnnexinV and Topro.

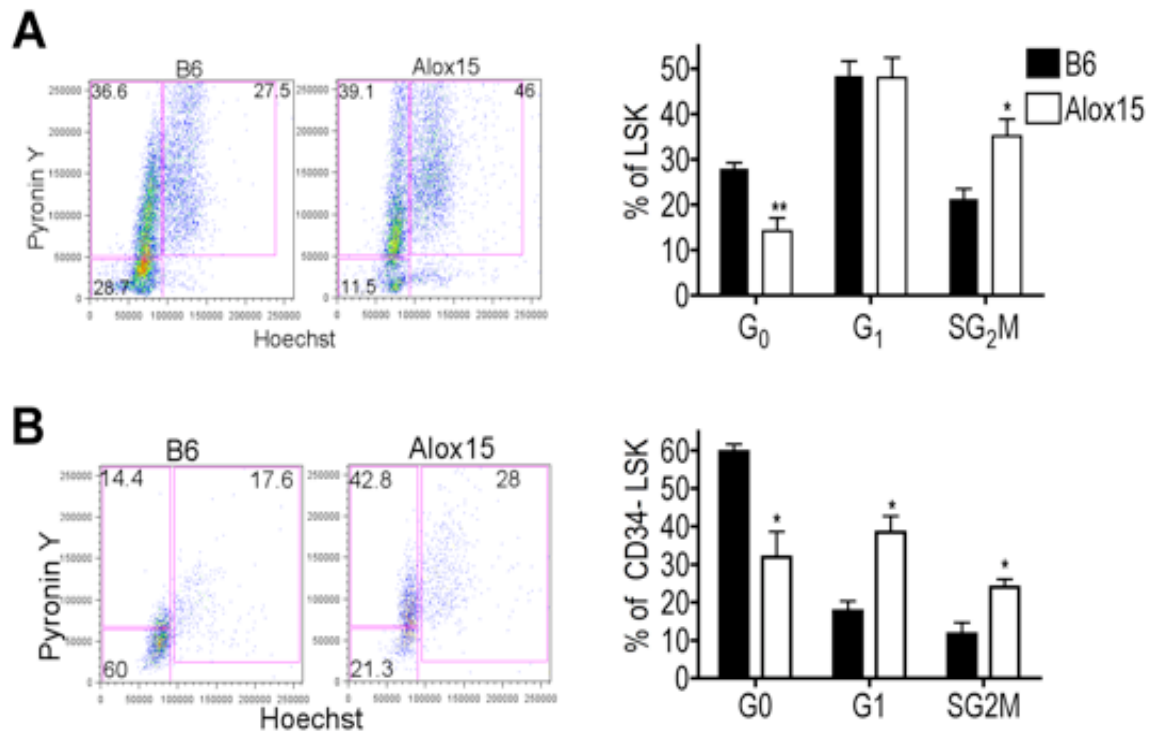


Figure 26. Increased proliferation of Alox15 HSC compared to B6.

Flow cytometric analysis by incorporation of Hoechst 33342 and PyroninY into DNA and RNA respectively of cells in the (A) total LSK population and (B) CD34-LSK gated population. Shown are a representative flow cytometric analysis and a summary of 4 experiments. * $p < .05$, ** $p < .01$.

Defective self-renewal in Alox15 HSC

We tested whether 12/15-LOX regulates self-renewal capacity using two approaches. In the first, we compared the self-renewal capacity of Alox15 and B6 HSCs in serial transfer assays in the **absence** of competitors. B6 and Alox15 BM were transplanted into lethally irradiated mice. After 16 weeks, the bone marrow was isolated and transplanted into a second lethally irradiated recipient (Figure 27A). Alox15 BM was able to reconstitute lethally irradiated primary recipient mice but recapitulated the defects in Alox15 mice described above, compared to the hematopoietic profile of primary recipients of B6 BM (Figure 14). However, when BM cells from primary recipients were serially transferred, there was a marked reduction in the survival of secondary recipients of Alox15 BM compared to B6 (Figure 27B). The morbidity was not due to development of MPD because the secondary recipients had comparable levels of neutrophils (Figure 27C). These data demonstrate that Alox15 HSCs have a decreased capacity to self-renew compared to wild-type HSC.

In the second approach we compared functionality of B6 and Alox15 HSCs following treatment with 5-FU (200 mg/kg, i.p.), which depletes the mature hematopoietic compartment resulting in division of HSCs. During competitive reconstitutions, HSCs must be able to home to a bone marrow environment that is altered after irradiation. The 5-FU approach has an advantage because it tests the ability of HSCs to self-renew in their native niches. HSC self-renewal capacity was assessed by determining the ratios of LSK to myeloid progenitors (MP) and CLPs at day 4 post-injection, when the HSC are expected to be cycling in

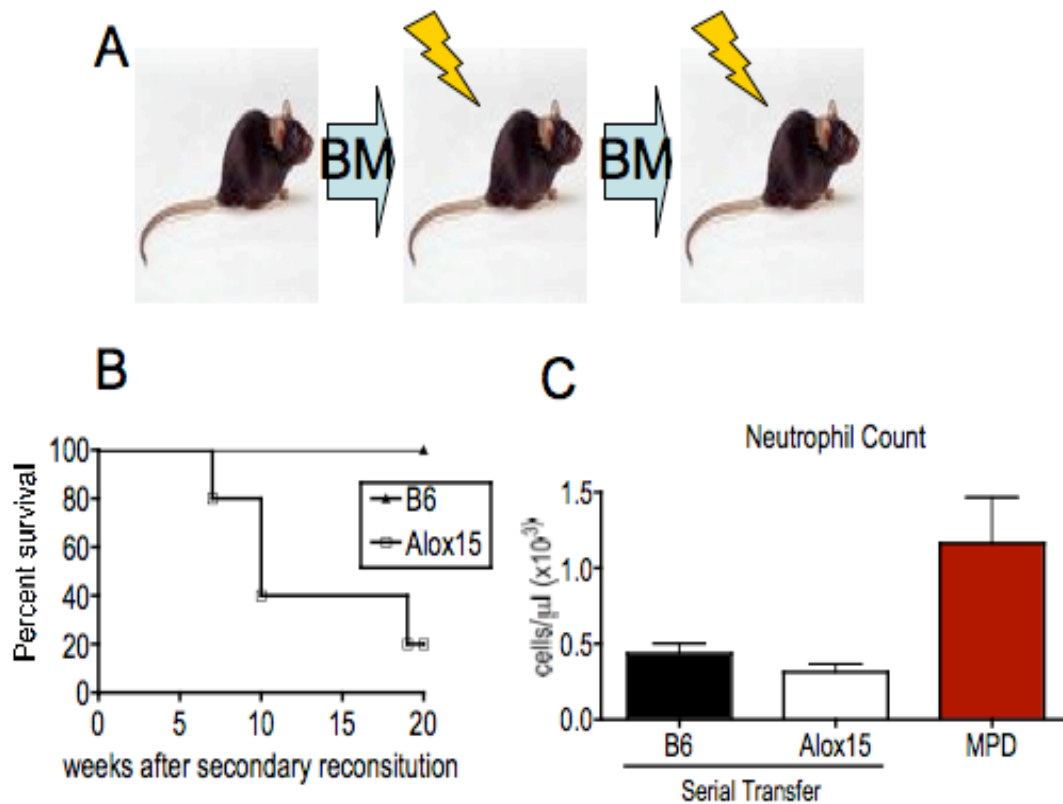


Figure 27. 12/15-LOX regulates self-renewal of HSC. (A) Schematic of serial reconstitution of WT or Alox15 BM. (B) Kaplan-Meier morbidity curve demonstrating decreased survival of secondary recipients of Alox15 BM compared to WT. (C) Neutrophil count in secondary B6 and Alox15 BM recipients demonstrating that the mortality in secondary recipients is not due to development of MPD.

order to replenish the hematopoietic compartment. If the ratios of CLP/LSK or MP/LSK are increased in 5-FU treated Alox15 BM compared to 5-FU treated wild-type controls, it would indicate a preference for Alox15 HSCs to differentiate rather than to self-renew while a reduced ratio indicates a preference for Alox15 HSCs to self-renew rather than differentiate (Figure 28). On day 4 post-5-FU treatment, Alox15 BM contained a greater percentage of differentiated cells compared to wild-type including an increase in the percentage of MP and increased percentage and numbers of CLPs (Figure 29A-C). Moreover, the ratios of MP/LSK, and CLP/LSK were increased compared to wild-type, demonstrating a decreased capacity for Alox15 HSC to self-renew and an asymmetric division skewed toward differentiation (Figure 29A). B6 and Alox15 LSK displayed similar resistance to 5-FU per se because at day 2 post-5-FU treatment, there were no differences in the numbers or percentages of LSK, MP or CLP. Furthermore, by day 8 post 5-FU treatment, the LSK subset in B6 had expanded while Alox15 exhibited a marked reduction in number and percentage of LSK. (Figure 29B-C). The reduction in LSK in BM of 5-FU-treated Alox15 mice on day 8 may be due to exhaustion of the capacity of HSC to self-renew as required to maintain the populations of LSK and progenitors. 5-FU-treated Alox15 mice also exhibited decreased survival; 87.5% (7/8) of Alox15 mice succumbed to treatment compared to 12.5% (1/8) for B6 mice (Figure 29D). The cause of death of the 5-FU-treated Alox15 mice appeared to be severe anemia, as the HCT at day 8 post-treatment was $14.52\% \pm 4.81\%$ for Alox15 mice versus $32.53\% \pm 8.08\%$ for B6 mice. The findings of increased proliferation but decreased numbers of

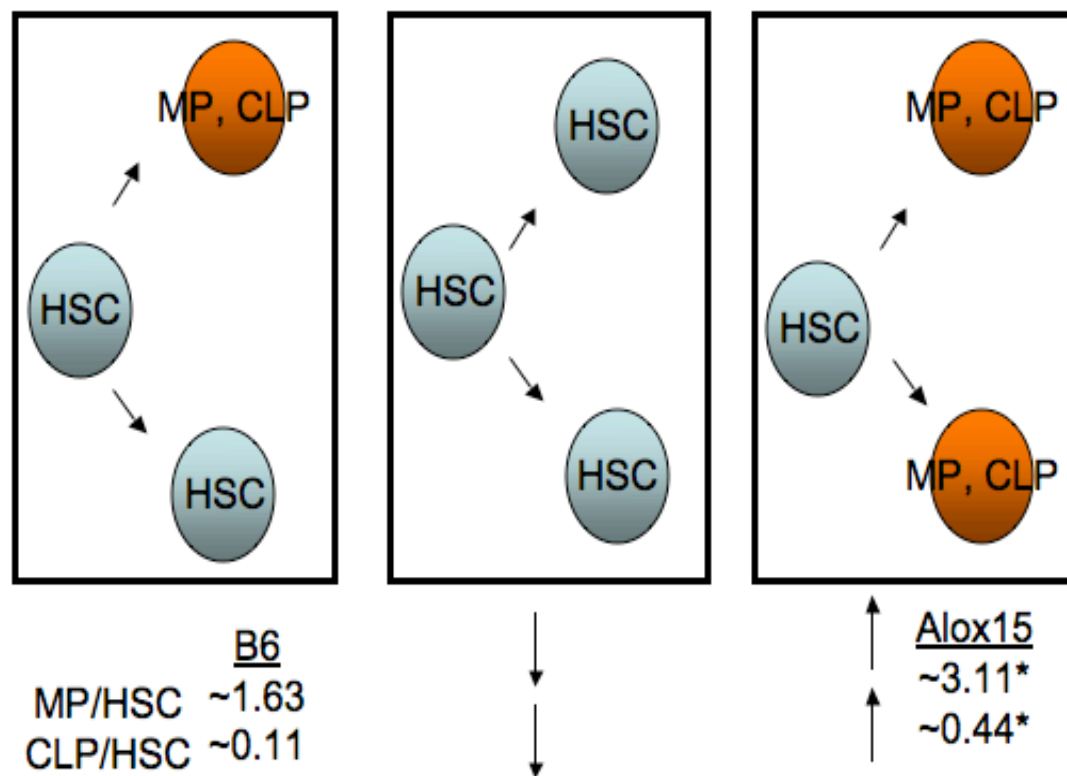


Figure 28. Schematic of enhanced differentiation and decreased self-renewal in Alox15 HSCs. Schematic of proliferation of HSC following 5-FU treatment and expected ratios of MP/HSC and CLP/HSC post-treatment with 5-FU. Alox15 exhibits an increased ratio of progenitors to HSCs.

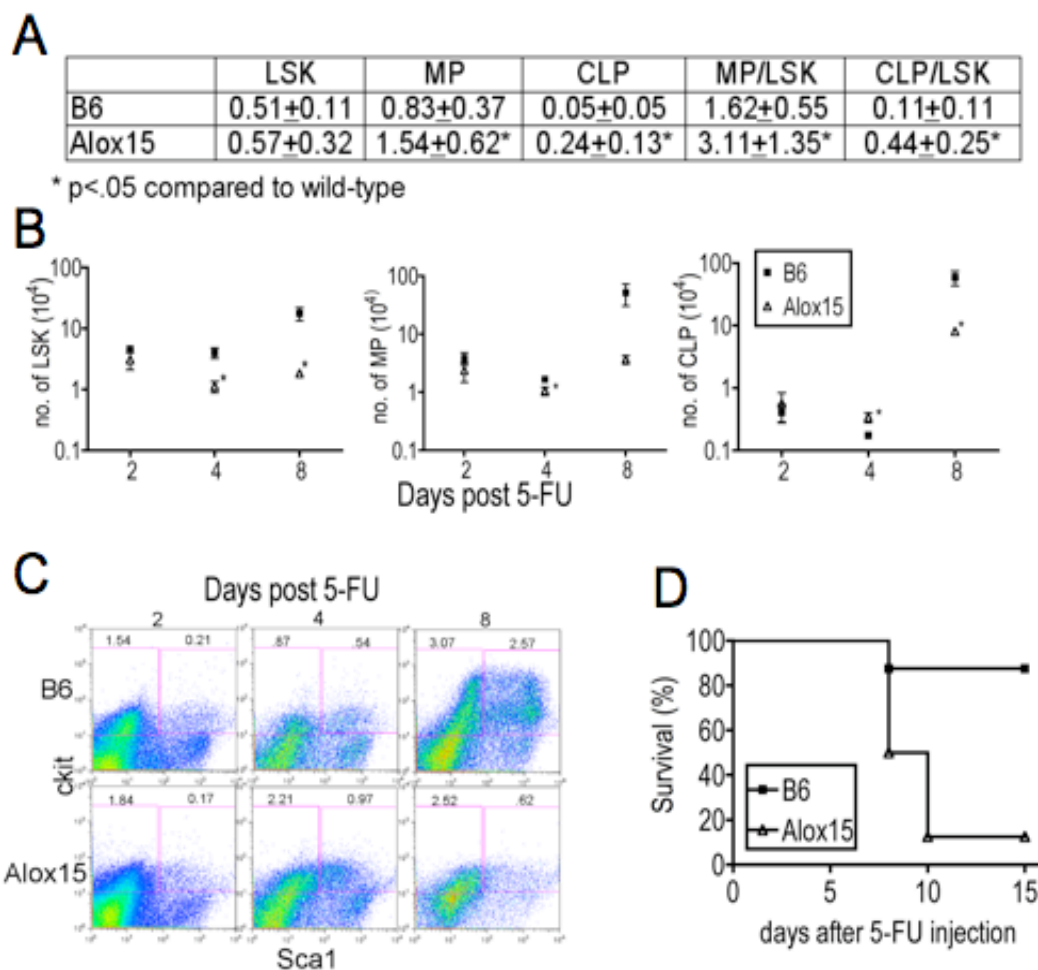


Figure 29. Alox15 HSCs exhibit decreased self-renewal capacity. (A) Increased ratio of myeloid progenitors (MP:Lin-Sca1-cKit+) and CLP to LSK quantified by flow cytometry day 4 following treatment with 200 mg/kg 5-FU indicate a defect in self-renewal capacity of Alox15 LSK compared to B6. (n=9); (B-D) Kinetics of expansion of LSK and progenitor populations in 5-FU treated B6 and Alox15 mice demonstrating loss of the LSK subset in Alox15 mice by day 8. (B) Numbers of LSK, MP and CLP and (C) representative flow cytometric analyses at times indicated of gated Lin- cells with percentages of progenitor and LSK subsets amongst total cells. (D) Kaplan-Meier plot demonstrating decreased survival of 5-FU treated Alox15 mice compared to B6 (n=8)

LT-HSC in Alox15 mice, decreased survival of recipients that received serially transferred Alox15 bone marrow and the increased ratios of Alox15 progenitor/LSK during 5-FU assays, when taken together, effectively demonstrate that 12/15-LOX regulates HSC self-renewal.

Defective self-renewal of Alox15 HSC is associated with reduction 12/15-LOX-dependent generation of lipid mediators and ROS

In order to determine which products of 12/15-LOX activity mediate HSC function, we compared levels of ROS and lipid metabolites in B6 and Alox15 progenitors. Purified Alox15 LSK isolated by cell sorting exhibited increased basal ROS levels *ex vivo* using the oxygen-sensitive CM-H₂DCFDA probe compared to B6 (Figure 30A). Because ROS levels are known to be decreased in LT-HSC, the increase in basal ROS levels is likely a reflection of the decreased numbers of LT-HSC within the Alox15 LSK (Figure 24) (Jang and Sharkis, 2007). Interestingly, however, wild-type LSK exhibited a greater fold increase in ROS compared to Alox15 after loading with exogenous AA indicating that 12/15-LOX-mediated ROS production is in fact defective in Alox15 LSK (Figure 30B). Hence, we speculate that 12/15-LOX-mediated ROS production may contribute to 12/15-LOX signaling in HSCs. However, in the HSC it is difficult to restore 12/15-LOX dependent ROS without affecting basal ROS, which is increased due to other oxidases such as other enzymes involved in lipid metabolism, NADPH-oxidases and mitochondria. In the future, it would be interesting to elucidate the effects of these other oxidases in Alox15 HSC that may contribute to the increased basal ROS levels and may also effect HSC

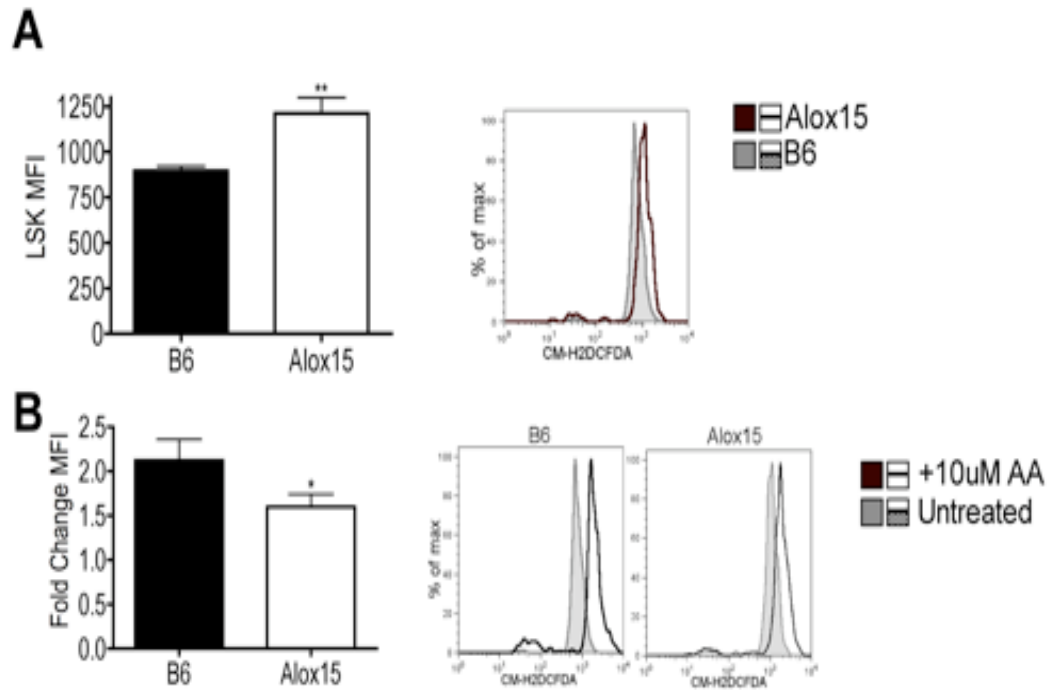


Figure 30. Reduction in 12/15-LOX-generated ROS in Alox15 HSC. (A) Increased basal ROS in Alox15 HSC. B6 and Alox15 LSK were isolated by cell sorting and analyzed for ROS formation using the ROS sensitive indicator CM-H₂DCFDA and flow cytometry. Shown are a summary of Mean Florescence Intensity (MFI) of n=5 in 2 independent experiments and a representative flow cytometric analysis. Shaded-B6; Open-Alox15 (B) B6 and Alox15 LSK were loaded with 10uM AA prior to measuring ROS. Shown are a summary of n=4 in 2 independent experiments as fold increase MFI compared to unstimulated cells and representative flow cytometric analyses. Shaded,unstimulated; Open,Stimulated.

function. In Chapter 3, we will elucidate that one effect of 12/15-LOX-generated ROS is to regulate ICSBP/IRF-8 transcription factor nuclear accumulation through redox-dependent signaling.

We also compared the generation of 12/15-LOX-mediated lipid metabolites between B6 and Alox15 BM and progenitors. B6 and Alox15 BM or Lin⁻ cells enriched by column purification were loaded with 10 μ M AA and lipid products were detected using mass spectroscopy (Figure 31A). Alox15 BM exhibited decreased 12(S)-HETE and 15(S)-HETE production while Alox15 Lin⁻ cells produced less 12(S)-HETE and 13(S)-HODE compared to B6 (Figure 31B). These differences were specific for 12/15-LOX products as there were no differences in the 5-LOX product 5(S)-HETE. Interestingly, Alox15 BM also exhibited decreased levels of the novel recently described 15(S)-HETE derivative 15-oxo-ETE, reported to inhibit proliferation in HUVEC cells (Wei et al., 2009). We speculate that 12/15-LOX lipid product signaling by 12(S)-HETE and 13(S)-HODE may mediate the effects of 12/15-LOX on HSC function.

As described in the introduction, 12/15-LOX produces multiple products found in both the cell membrane, in the cytoplasm and released outside the cell. The mass spectroscopy assay used only measures specific lipid products that are released into the supernatant and it is likely that other 12/15-LOX-generated products not measurable by our assay play a role in cell signaling that regulates HSC function. Moreover, products of 12/15-LOX often act in combination, which makes testing a single 12/15-LOX in isolation difficult to interpret. Product re- diversion in the absence of 12/15-LOX occurs and another product of

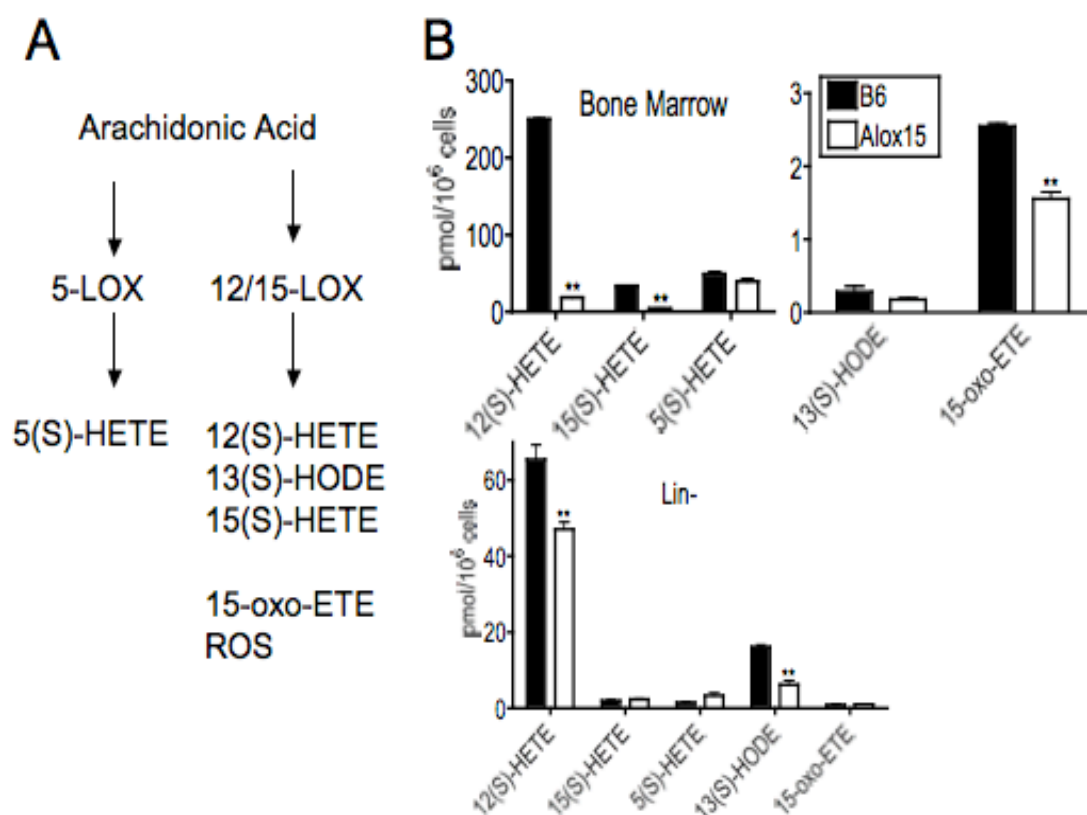


Figure 31. Alox15 HSCs have select defects in 12/15-LOX generated lipid mediators. (A) Overview of Lipoxigenase mediated catalysis of arachidonic acid (B) Lipid product formation in B6 and Alox15 BM and Lin⁻ cells after stimulation with 10 μ M AA; *p<.05, **p<.01 compared to wild-type.

different AA-metabolizing enzyme such as 5-LOX may in fact regulate HSC function. Lastly, lipid metabolites are very short-lived and stable derivatives of the 12/15-LOX products may be needed to test their ability to regulate HSC function.

Despite these challenges, we tested the ability of 12/15-LOX products, 12(S)-HETE and 15(S)-HETE, to alter HSC proliferation *in vitro*. BM cells from Alox15 mice were isolated and treated with 12(S)-HETE and 15(S)-HETE in varying concentrations for 30 minutes or overnight prior to cell cycle analysis. Both 12(S)-HETE and 15(S)-HETE increased the percentage of cells in G₀ and decreased the percentage of cycling cells in the total BM population (Figure 32A). These effects were not dose-dependent and occurred at the lowest dose of 1 μ M for both 12(S)-HETE and 15(S)-HETE. However, neither 12(S)-HETE nor 15(S)-HETE alone had any effect on the proliferation of LSK cells (Figure 32B-C). Therefore, 12(S)-HETE nor 15(S)-HETE in isolation regulates HSC proliferation, rather 12/15-LOX likely regulates HSC with greater complexity. Products of 12/15-LOX may also regulate mature cells differently than LSK, as 12(S)-HETE and 15(S)-HETE both effected the proliferation of the total BM population but not the LSK cells. Moreover, to regulate their cell division *in vivo* HSC rely on signals provided by their niche, which is absent from this *in vitro* assay.

12/15-LOX regulates ICSBP/IRF-8 nuclear accumulation and consequently canonical Wnt signaling in hematopoietic progenitors

We previously reported that 12/15-LOX regulates ICSBP/IRF-8 nuclear accumulation in peritoneal macrophages and Mac1⁺Gr1⁺ splenocytes

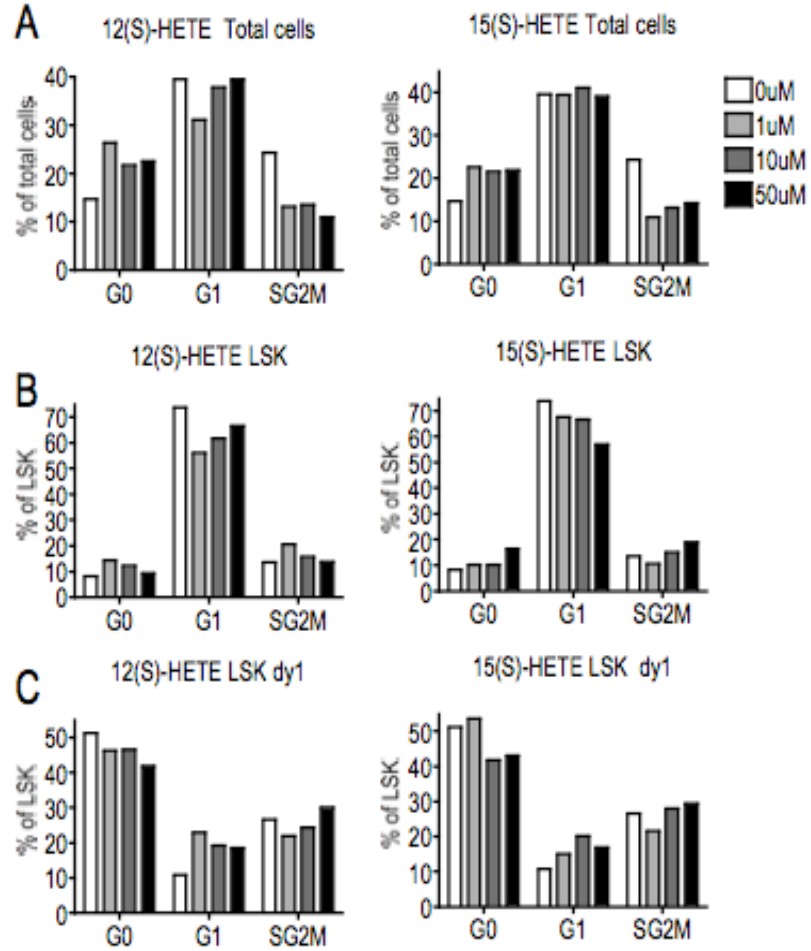


Figure 32. 12(S)-HETE and 15(S)-HETE inhibit proliferation of bone marrow cells but not HSCs *in vitro*. BM from Alox15 mice was isolated and treated with varying concentrations of 12(S)-HETE and 15(S)-HETE for 30 minutes (A-B) or overnight (C) prior to staining for cell cycle analysis. Shown are data gated on total cells (A) and LSK (B-C).

(Middleton et al., 2006a; Middleton et al., 2006b). ICSBP/IRF-8 is also reduced in aging stem cells and its targets, including Dab2, are involved in signaling pathways implicated in HSC function (Hocevar et al., 2003; Stirewalt et al., 2008). Therefore, we investigated whether 12/15-LOX regulates ICSBP/IRF-8 in hematopoietic progenitors. ICSBP/IRF-8 protein in nuclear extracts of B6 and Alox15 Lin⁻cKit⁺ cells enriched by column purification was quantified by immunoblot *ex vivo*. The levels of nuclear ICSBP/IRF-8 protein were decreased in Alox15 progenitors compared to B6 (Figure 33A) due to dysregulation at a post-translational level as levels of ICSBP/IRF-8 total protein (MPER lysates) and mRNA were not statistically different (Figure 33B-C).

To determine whether the decrease in ICSBP/IRF-8 nuclear accumulation affected the expression of ICSBP/IRF-8 target genes in HSC, we analyzed mRNA levels of known ICSBP/IRF-8-regulated genes using Q-PCR in B6 and Alox15 LSK enriched by sorting. ICSBP/IRF-8 is known to negatively regulate Dab2 and positively regulate Nf-1, Blimp1 and p15INK4b (Rosenbauer et al., 2002; Schmidt et al., 2004; Zhu et al., 2004). Consistent with this, Dab2 mRNA was increased while Nf-1, Blimp1 and p15INK4b mRNA were decreased in Alox15 compared to wild-type LSK (Figure 33D).

We also analyzed expression of other genes that regulate HSC function not believed to be directly regulated by ICSBP/IRF-8 including Hes1, Bmi, Hoxb4 and Gfi-1. Of these genes, only Hes1 was significantly decreased in Alox15 compared to wild-type LSK (Figure 33D). Because Hes1 is also decreased when canonical Wnt signaling is inhibited (Fleming et al., 2008) and because the

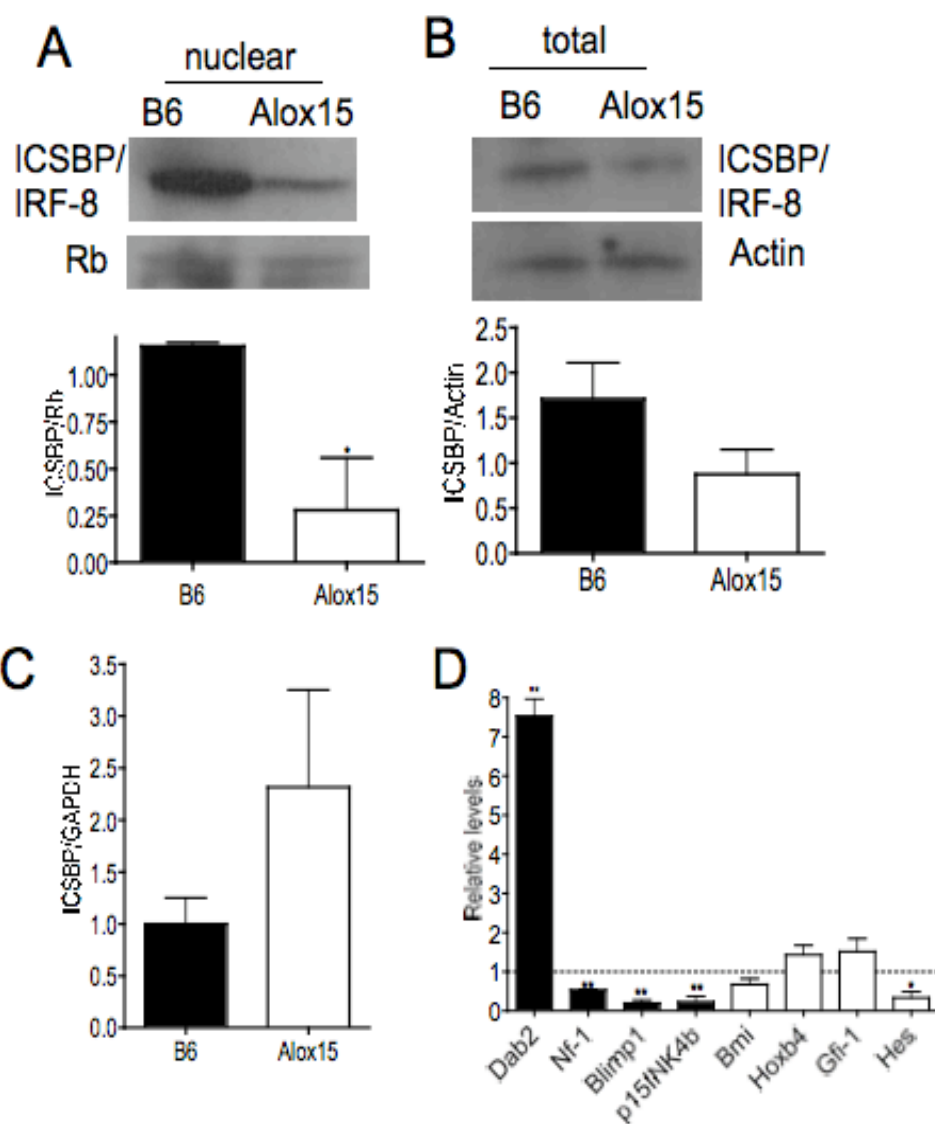


Figure 33. 12/15-LOX regulates ICSBP/IRF-8 in HSC. (A) Immunoblot of nuclear extracts from BM Lin-cKit⁺ cells demonstrating decreased nuclear accumulation of ICSBP/IRF-8 in Alox15 progenitors. (B-C) Dysregulation of ICSBP/IRF-8 protein is post-transcriptional. Similar quantities of ICSBP/IRF-8 (B) total protein lysates and (C) mRNA B6 and Alox15 Lin-cKit⁺ cells (n=3). (D) Q-PCR of mRNAs of ICSBP/IRF-8-regulated genes (black bars) and genes known to regulate HSC self-renewal but not regulated by ICSBP/IRF-8 (white bars) in sorted B6 and Alox15 LSK presented as average fold change from B6 (n=3).

ICSBP/IRF-8 target Dab2 negatively regulates canonical Wnt signaling by inhibiting disheveled and stabilizing axin (Hocevar et al., 2003; Jiang et al., 2008), we investigated whether 12/15-LOX regulates canonical Wnt signaling. We first determined that Dab2 protein was increased in Alox15 Lin⁻cKit⁺ cells (Figure 34A). Because increased Dab2 leads to degradation of downstream mediators of canonical Wnt signaling including β -catenin, we measured β -catenin levels in wild-type and Alox15 progenitors. Indeed, β -catenin protein was decreased in total lysates of Alox15 Lin⁻cKit⁺ progenitors compared to wild-type (Figure 34B). These data implicate 12/15-LOX as a novel regulator of canonical Wnt signaling. Canonical Wnt signaling is known to regulate HSC quiescence and self-renewal (Fleming et al., 2008; Luis et al., 2009; Zhao et al., 2007) and therefore the dysregulation of the Wnt signaling pathway including β -catenin, may be at least one important mechanism underlying the reduced quiescence and self-renewal capacity in Alox15 HSC.

The PI3-K pathway is another signaling pathway implicated in HSC function. We have previously shown that phosphorylated Akt, which is downstream of PI3-K was increased in developing Alox15 BM and that PI3-K regulated nuclear accumulation of ICSBP/IRF-8 in Alox15 splenocytes (Middleton et al., 2006b). Moreover, mice with activated PI3-K due to deletion of PTEN exhibited HSC defects including loss of quiescence and developed leukemia. These effects were mediated downstream by mammalian target of rapamycin (mTOR) signaling. Treatment of these mice with the mTOR inhibitor rapamycin reduced proliferation of PTEN-deficient HSC (Yilmaz et al., 2006). Therefore,

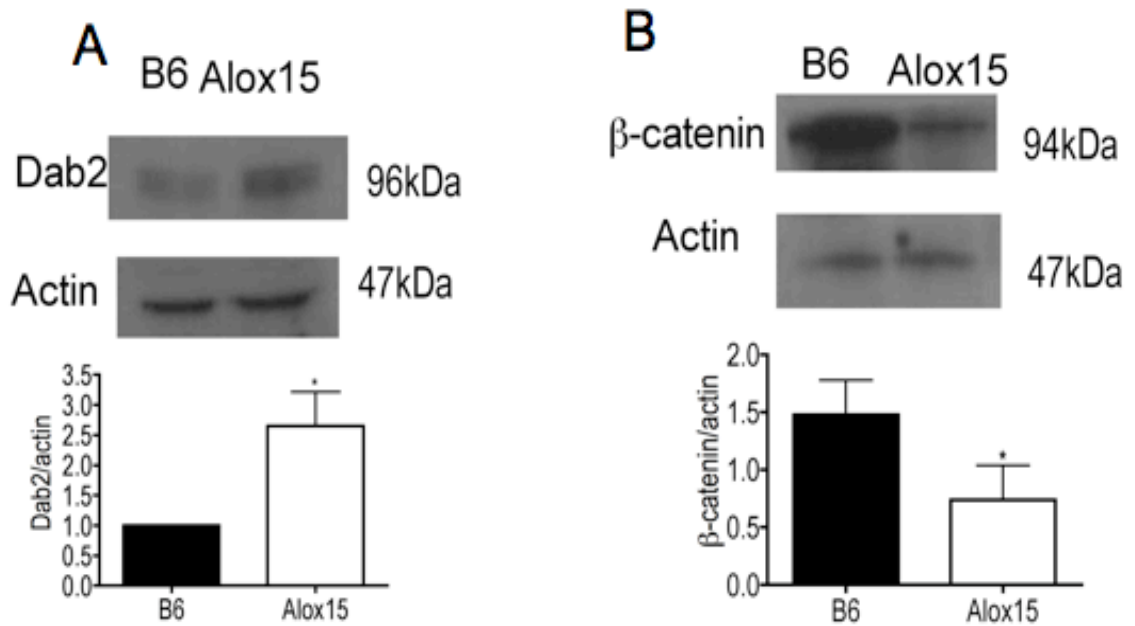


Figure 34. Decreased canonical Wnt signaling in Alox15 Lin⁺cKit⁺ progenitors.

Immunoblot (top) and densitometric quantification of (A) Dab2, an inhibitor of Wnt signaling, and (B) β-catenin, a downstream mediator of canonical Wnt signaling, in lysates of B6 and Alox15 Lin⁺cKit⁺ cells (n=4); *p<.05, **p<.01 compared to wild-type.

we speculated that activated PI3-K signaling, specifically mTOR, may regulate the defective HSC function in Alox15 mice. We tested whether treatment with rapamycin reduced HSC proliferation in Alox15 mice. Treatment of Alox15 mice with rapamycin for seven days decreased the proliferation amongst the total Alox15 BM population (Figure 35A) demonstrating that the biological activity of rapamycin and that the enhanced proliferation in mature Alox15 cells may be due in part to mTOR signaling. On the other hand, Alox15 LSK displayed no difference in proliferation in the presence of rapamycin (Figure 35B). Therefore, the increased proliferation in Alox15 LSK is not due to the PI3-K-AKT-mTOR pathway.

In order to directly address whether β -catenin regulates HSC function in Alox15 mice, we attempted to introduce a murine stem cell retrovirus (MigR) containing non-degradable β -catenin into Alox15 BM. However, retroviral transduction of Alox15 cells was challenging. Treatment of Alox15 mice with 5-FU caused depletion of the LSK and progenitors (Figure 29). Therefore, we tested the feasibility of retroviral transduction of fetal liver cells, which do not need treatment with 5-FU. Alox15 fetal liver cells were retrovirally transduced at high levels initially. However, lethally irradiated recipients that received Alox15 retrovirally transduced cells exhibited low survival rates, while all of the recipients of B6 retrovirally transduced cells survived (Figure 36A). The few surviving recipients of Alox15-transduced cells exhibited low levels of GFP+ Mig retrovirus (A) while the B6 retrovirally transduced cells were present at high numbers (Figure 36A). Furthermore, surviving recipients of Alox15 transduced cells

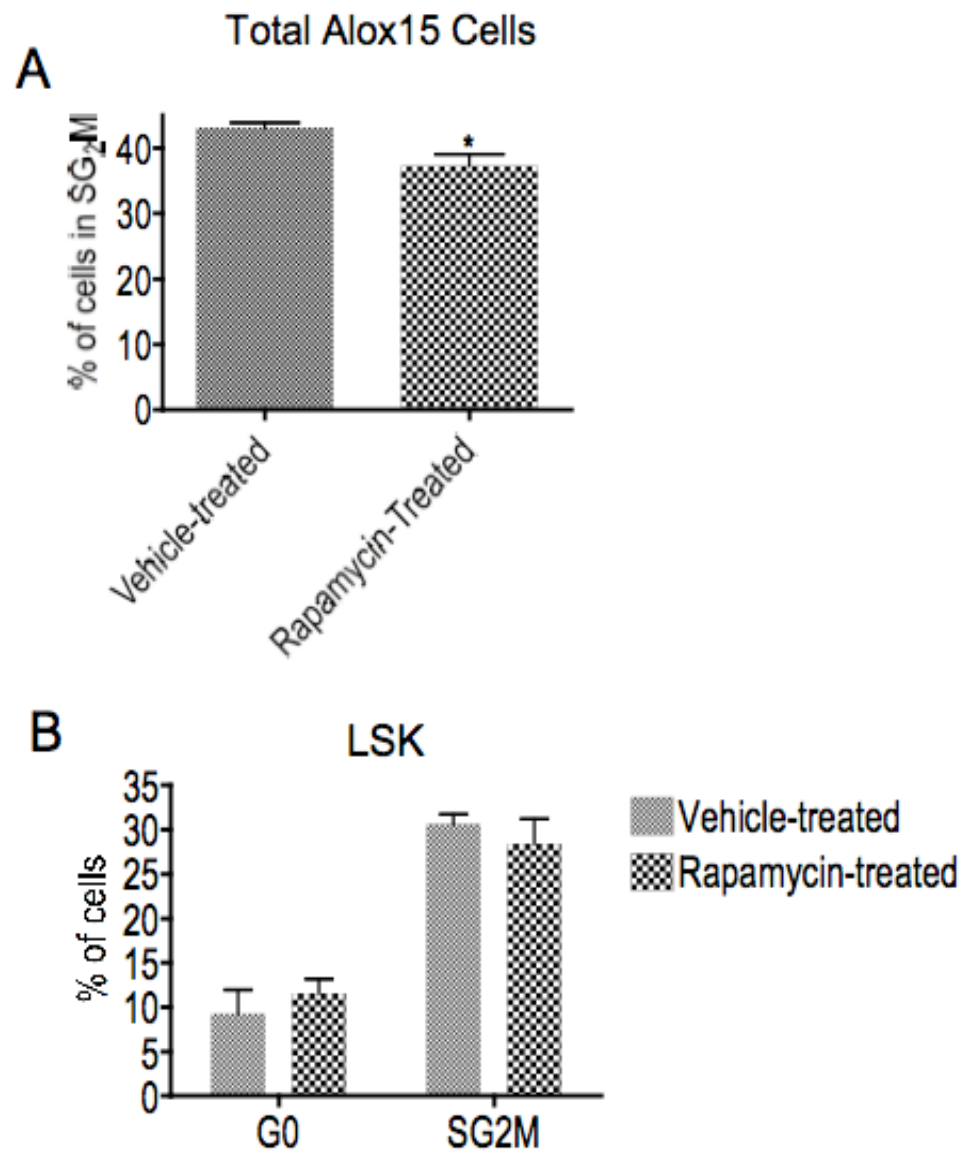


Figure 35. Proliferation of Alox15 LSK is not dependent on mTOR. Alox15 mice were treated with rapamycin or vehicle control i.p. daily. After 7 days, BM was isolated and percentage of proliferating cells in the total BM (A) or LSK (B) were determined by Hoechst staining and multicolor flow cytometry. * $p < 0.05$

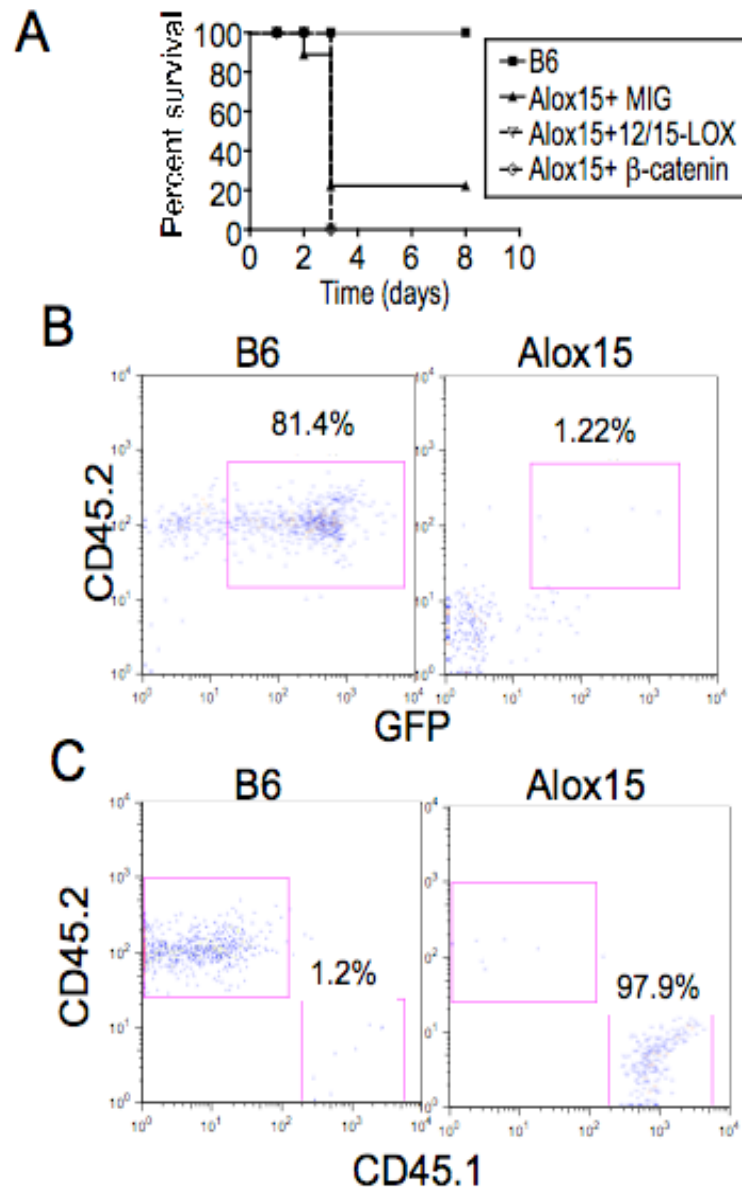


Figure 36. Alox15 cells exhibit sensitivity to retroviral transduction. B6 and Alox15 fetal liver cells were retrovirally transduced with Mig retrovirus and then transferred into lethally irradiated congenic recipient host. (A) Survival curve demonstrating decreased survival of recipients receiving retrovirally transduced Alox15 cells $n=10$ for MigR, $n=5$ for MigR containing 12/15-LOX or β -catenin (B-C) After 8 weeks, peripheral blood was isolated from surviving recipients and analyzed using flow cytometry for presence of retrovirus by GFP (B) or presence of recipient cells by CD45.1 (C). Shown is a representative experiment.

but not recipients of B6 retrovirally transduced cells, exhibited an expansion of rare host cells (Figure 36B). Thus, it appears that Alox15 cells exhibit greater sensitivity to retroviral transduction and do not survive/expand after transfer into irradiated recipients. Lowering the dose of irradiation to the host or the addition of competitor B6.SJL cells improved survival of recipients but did not improve recovery of Alox15 retrovirally transduced cells. The retroviral reconstitution in Alox15 HSCs needs to be further optimized in future studies to directly address the pathway whereby 12/15-LOX regulates HSC.

Implications

These studies establish the importance of unsaturated fatty acid metabolism in hematopoiesis by demonstrating that Alox15 mice have a cell-autonomous defect in hematopoietic development. Depletion of 12/15-LOX results in a primary defect in HSC function. 12/15-LOX directly regulates the number, proliferation and function of LT-HSCs. Furthermore, Alox15 HSCs exhibit a decreased capacity to self-renew. The defects in Alox15 HSCs are associated with a selective decrease in 12/15-LOX-mediated ROS and lipid metabolites and are at least partly due to dysregulation of the transcription factor ICSBP/IRF-8 and its downstream targets and to disruption of canonical Wnt signaling.

Because Alox15 HSCs exhibit decreased quiescence, the striking phenotype observed during the 5-FU assays at day 4 may be partially due to increased sensitivity of Alox15 LSK to 5-FU. However, the sensitivity of 5-FU at day 2 is similar between B6 and Alox15 LSK, suggesting that 5-FU is not

acting directly on Alox15 LSK. Moreover, the increased numbers of Alox15 CLP at day 4 compared to B6 demonstrate that Alox15 LSK are skewed in favor of differentiation rather than self-renewal. The increased ratio of progenitors compared to LSK at day 4, support the possibility that the exhaustion in the LSK population is at least partly due to a decrease in self-renewal. The conclusion that 12/15-LOX regulates self-renewal is also supported by the defect of Alox15 BM cells during serial reconstitution assays and by depletion of the LT-HSC population in Alox15 BM.

ROS, which are functional by-products of 12/15-LOX activity (Conrad, 1999), are known to regulate HSC function. Because LT-HSCs exhibit decreased ROS (Jang and Sharkis, 2007), the finding that Alox15 LSK exhibit increased basal levels of ROS is likely a reflection of the reduction in LT-HSCs amongst Alox15 LSK. Furthermore, basal ROS levels in HSCs may be determined by other oxidases, such as NADPH-oxidase (Piccoli et al., 2007). In any case, increased levels of ROS may contribute to the defect in HSC function and self-renewal (Ito et al., 2006; Miyamoto et al., 2007; Tothova et al., 2007; Jang and Sharkis, 2007). Moreover, ROS are known to regulate cell cycle progression (Iiyama et al., 2006) and hence may in fact contribute to the increased cycling in Alox15 LSK. However, we have demonstrated that 12/15-LOX-mediated ROS production is defective in Alox15 LSK and hence may contribute to 12/15-LOX signaling in HSCs.

Deficiency in 12/15-LOX also resulted in a marked reduction in the generation of select 12/15-LOX lipid mediators by hematopoietic progenitors.

12(S)-HETE and 13(S)-HODE were decreased while similar levels of 15(S)-HETE and the 5-LOX product 5(S)-HETE were produced by Alox15 compared to B6 progenitors. On the other hand, in Alox15 total BM cells there were decreased 12(S)-HETE and 15(S)-HETE but similar levels of 13(S)-HODE. The differences in product formation between BM cells and the enriched progenitors are likely due to the preference of murine 12/15-LOX to synthesize 12(S)-HETE rather than 15(S)-HETE and differences in substrate (AA vs linoelic acid) availability.

Lipid metabolites of 12/15-LOX may regulate HSC function intrinsically either individually or in combination and may act on multiple signal transduction pathways including activation of protein kinase C by 12(S)-HETE, and MAPK by 13(S)-HODE (Conrad, 1999). Some 12/15-LOX products function by incorporation into cell membranes, which can alter phosphoinositol (Girton et al., 1994) and receptor signaling (Takata et al., 1994). Moreover, 12/15-LOX products directly influence gene transcription by binding and activating transcription factors such as PPAR- γ (Huang et al., 1999). Recent studies have shown that the recently described 15(S)-HETE derivative 15-oxo-HETE inhibits HUVEC proliferation (Wei et al., 2009). Further studies are required to determine how 12/15-LOX lipid products regulate HSC proliferation and function.

Interestingly, fatty acid metabolism mediated by other enzymes that also target AA as their substrate have been implicated in normal and leukemic HSC function. The cyclooxygenase product prostaglandin E₂ is known to signal extrinsically through its receptor on HSC to promote self-renewal by enhancing canonical Wnt signaling and β -catenin accumulation (North et al., 2007)

(Goessling et al., 2009). Prostaglandin E₂ has also been shown to regulate HSC progression into cell cycle (Hoggatt et al., 2009). In addition, it was recently reported that 5-lipoxygenase-deficient mice are protected from BCR-ABL-induced CML through inhibition of leukemic stem cell differentiation. In this study, we demonstrate a seemingly opposing role for 12/15-LOX since 12/15-LOX promotes self-renewal of normal HSC and suppresses development of myeloid leukemia (Middleton et al., 2006b). Because cyclooxygenase, 5-LOX and 12/15-LOX share AA as a substrate, it will be interesting in the future to investigate whether substrate availability or substrate redirection may contribute to the phenotypes observed in the HSC compartment in the absence of 12/15-LOX or 5-LOX.

We have demonstrated that 12/15-LOX regulates nuclear ICSBP accumulation in HSC. Intriguingly, ICSBP/IRF-8 has been proposed to play a role in HSC function as aging wild-type murine and human HSC have decreased levels of ICSBP/IRF-8 (Stirewalt et al., 2008). PU.1, a transcription factor that interacts with ICSBP/IRF-8 is required for the capacity of HSC to self-renew (Iwasaki et al., 2005). Both ICSBP/IRF-8 and PU.1 bind to the Dab2 promoter and ICSBP/IRF-8 inhibits Dab2 expression (Rosenbauer et al., 2002) (Figure 37). Dab2 functions as a negative regulator of canonical Wnt signaling by inhibiting dishevelled-3 and by stabilizing axin leading to degradation of mediators of canonical Wnt signaling including β -catenin (Hocevar et al., 2003; Jiang et al., 2008; Figure 34; Figure 37). Axin over-expression has been shown to negatively regulate HSC self-renewal (Reya et al., 2003). Other mediators of canonical

Wnt signaling including β -catenin have also been shown to regulate self-renewal, quiescence and function of HSCs (Nemeth et al., 2006; Zhao et al., 2007; Fleming et al., 2008; Luis et al., 2009). Consistent with these data, Dab2 gene and protein expression were amplified in Alox15 HSCs (Figure 37). Dab2 promotes β -catenin degradation leading to a decrease of β -catenin in Alox15 progenitors (Figure 37). The data presented indicate that 12/15-LOX may act upstream of ICSBP/IRF-8 to promote Wnt signaling and hence self-renewal by inhibiting transcription of Dab2. Therefore, 12/15-LOX is a novel regulator of canonical Wnt signaling.

HSC function and hematopoietic differentiation are related and disruptions in HSC function can lead to the development of leukemia; for example, in the floxed PTEN mouse (Yilmaz et al., 2006). Alox15 mice develop a chronic myelogenous leukemia-like MPD in 15% of the mice over the course of a year. It is possible that the disruption in HSC function contributes to the development of MPD in Alox15 mice (Middleton et al., 2006b). Dysregulation of ICSBP/IRF-8 and decreased 12/15-LOX activity, particularly levels of 12(S)-HETE, are also associated with human CML (Stenke et al., 1991; Takayama et al., 1983). The contribution of decreased lipid mediators to the leukemic state remains largely unknown, although eicosanoid products may induce apoptosis of myeloid leukemic cell lines (Li et al., 2005; Mahipal et al., 2007). Because of the development of a myeloid leukemia in Alox15 mice, we hypothesized that 12/15-LOX also regulates myelopoiesis which we will address in Chapter 3.

In summary, we have found that 12/15-LOX-mediated fatty acid

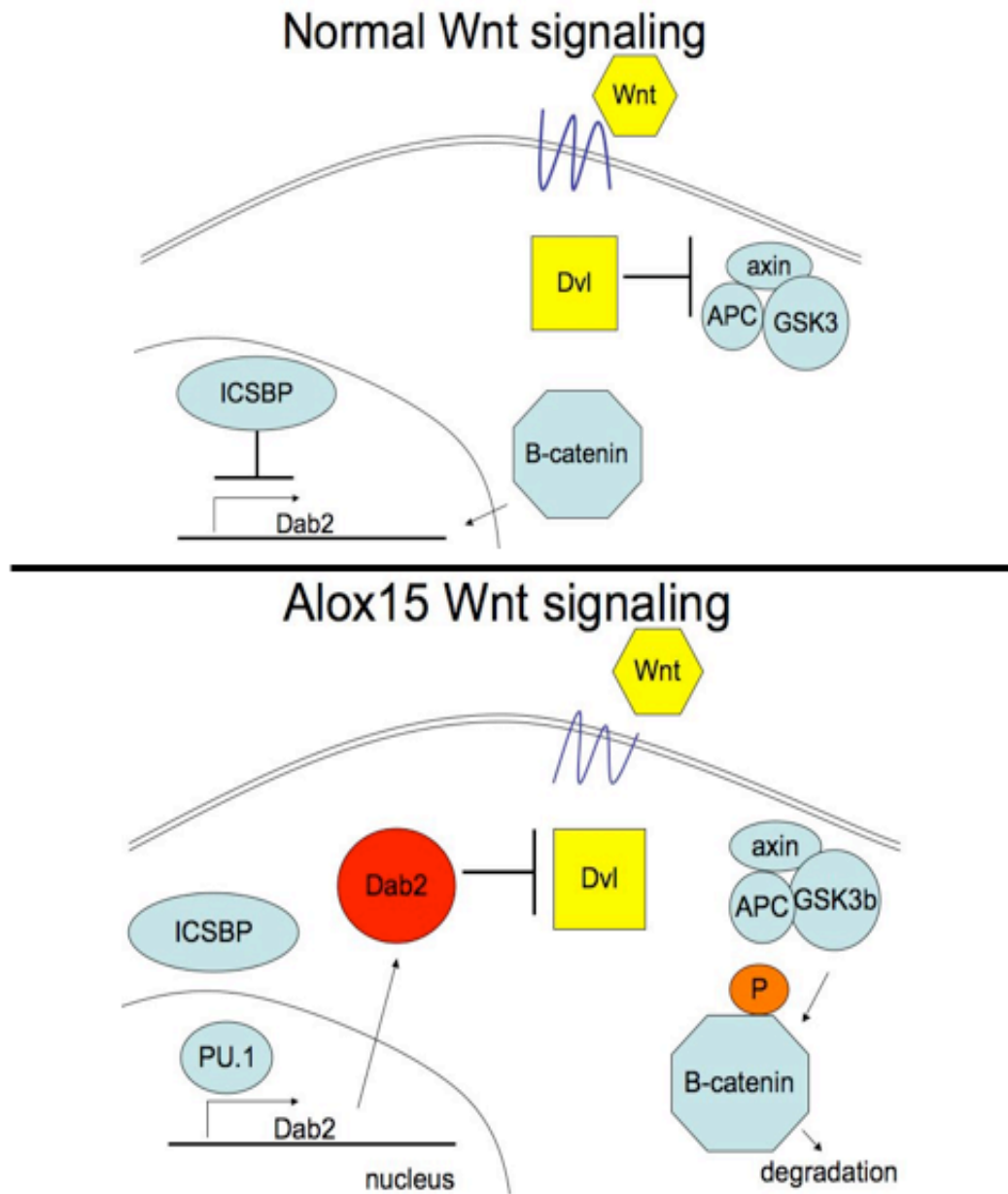


Figure 37. Schematic of canonical Wnt signaling in (A) wildtype cells and (B) dysregulated signaling in Alox15 HSC. Normally ICSBP represses Dab2 transcription, allowing canonical Wnt signaling and β -catenin accumulation to occur. Alox15 HSC exhibit enhanced Dab2 expression due to decreased ICSBP nuclear accumulation. Dab2 then inhibits canonical Wnt signaling by inhibiting Dvl and stabilizing the β -catenin destruction complex leading to decreased β -catenin levels.

metabolism regulates the asymmetric division of HSCs by promoting quiescence and self-renewal of LT-HSC. In the absence of 12/15-LOX, HSCs exhibit increased proliferation and a bias in their asymmetric division toward differentiation rather than self-renewal. Furthermore, we show that depletion of 12/15-LOX products is associated with a defect in nuclear accumulation of ICSBP/IRF-8 and subsequent canonical Wnt signaling. Furthermore, I define 12/15-LOX as a novel regulator of canonical Wnt signaling. The data in this chapter demonstrate that signaling downstream of fatty acid metabolism is an important mechanism whereby HSC function is mediated. Further understanding of these mechanisms may have implications for hematopoiesis, leukemia and hematopoietic aging.

CHAPTER 3: IRF-8-driven monopoiesis is dependent upon 12/15-lipoxygenase mediated redox signaling

Myelopoiesis is the process whereby myeloid cells, granulocytes and monocytes, are generated from HSCs. Hematopoietic differentiation from HSC gives rise to cells that are progressively more restricted in their potential fate as they commit to various lineages. For example, HSCs differentiate into MPP that propagate both myeloid and lymphoid cells (Wilson et al., 2007). Further differentiation of MPPs leads to the generation of CMP that can only sustain erythropoiesis by differentiating into MEP and myelopoiesis by differentiating into GMP, which in turn differentiate into either granulocytes or monocytes (Akashi et al., 2000). Although phenotypic profiles can be used to distinguish hematopoietic progenitors at various stages of differentiation, hematopoiesis occurs along a continuum so that phenotypically defined progenitor populations can exhibit heterogeneity with regard to their ability to commit and differentiate to alternative lineages.

During myelopoiesis, IRF-8 functions in conjunction with PU.1 to activate gene transcription that promotes monocytic differentiation while inhibiting gene transcription that promotes granulocytic differentiation (Tamura et al., 2000). Loss of IRF-8 transcriptional activity results in defects in monocytic development and enhanced granulocytic differentiation (Scheller et al., 1999; Tsujimura et al., 2002). Transcriptional activity of IRF-8 is dependent on its translocation to the nucleus from the cytoplasm and our lab previously showed that IRF-8 nuclear accumulation requires 12/15-lipoxygenase (12/15-LOX)-mediated signaling

(Middleton et al., 2006a; Middleton et al., 2006b). However, the mechanism whereby 12/15-LOX promotes nuclear accumulation of IRF-8 has not been elucidated. In this chapter, I present evidence which demonstrates that 12/15-LOX regulates ICSBP/IRF-8 by redox signaling and implicates SHP-2 as a redox sensitive factor.

Although much is known about the transcription factors that regulate myeloid cell specification, less is understood about the upstream signaling pathways required for these cell fate decisions. In this chapter, I present data which demonstrate that myelopoiesis is regulated by a novel 12/15-LOX-dependent signaling pathway. Little was previously known of the consequences of 12/15-LOX signaling during myelopoiesis. Although others reported that addition of 12(S)-HETE and 15(S)-HETE to human CD34⁺ progenitors decreased the numbers of granulocyte-macrophage colonies in methylcellulose assays (Desplat et al., 2000), the role of 12/15-LOX in regulation of granulocytic and monocytic lineage specification remained to be determined.

ROS generated as byproducts of 12/15-LOX-mediated lipid metabolism also function as second messengers by regulating cellular redox signaling. For instance, ROS generated by lipoxygenases can oxidatively modify proteins such as SHP-2 resulting in their inactivation (Pani et al., 2000; Chen et al., 2009a). Interestingly, SHP-2 negatively regulates ICSBP/IRF-8 transcriptional activity, known to promote monocyte differentiation (Huang et al., 2006; Tsujimura et al., 2002) and inhibition of SHP-2 results in decreased granulocyte differentiation (Jack et al., 2009). However, a role for ROS per se has not been previously

demonstrated in GMP or in the regulation of cell fate decisions.

Because monocyte numbers are reduced (Chapter 1) while the percentage of granulocytes is increased in Alox15 mice (Middleton et al., 2006b), I hypothesized that 12/15-LOX regulates myeloid cell fate decisions. In this chapter, I present data that establish that Alox15 myeloid cell development is skewed towards the granulocytic lineage at the expense of monopoiesis. Furthermore, the data indicate that the bias in cell fate is due to 12/15-LOX-dependent redox signaling that mediates nuclear accumulation of IRF-8 to drive monopoiesis and inhibit granulopoiesis.

Alox15 progenitors exhibit enhanced granulopoiesis and decreased monopoiesis

We previously demonstrated that 12/15-LOX-deficient Alox15 mice had reduced numbers of monocytes in the blood and increased percentage of granulocytes in the blood, spleen and bone marrow (BM) (Kinder et al submitted; (Middleton et al., 2006b). Therefore, we hypothesized that 12/15-LOX regulates myelopoiesis. Using real time PCR analysis of enriched progenitor subsets, we determined that 12/15-LOX was expressed at varying levels throughout myelopoiesis (Figure 38) with the highest levels of 12/15-LOX expression in CMP and MEP. Because these progenitors have erythroid potential, these data were consistent with the fact that 12/15-LOX products were involved in erythroid differentiation (Chapter 1; Kuhn and Brash, 1990; van Leyen et al., 1998). However, 12/15-LOX was also expressed in HSC and GMP at levels similar to Gr1⁺ splenocytes.

To test the hypothesis that 12/15-LOX regulates myelopoiesis, we

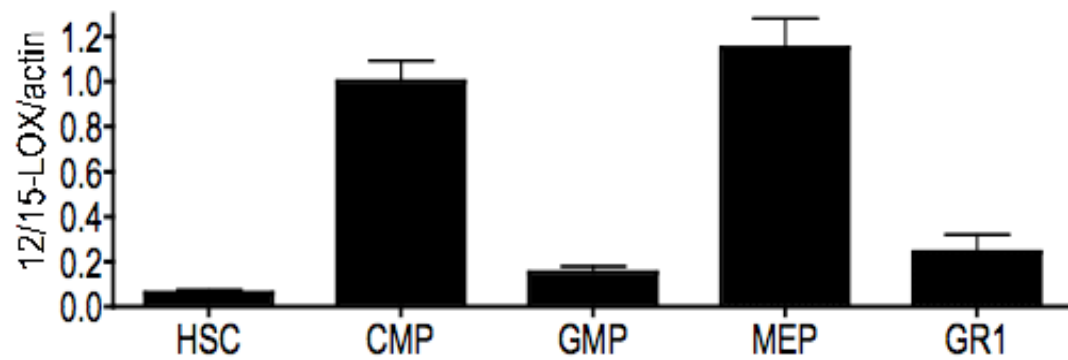


Figure 38. 12/15-LOX is expressed during myeloid differentiation. B6 HSC (Lin⁻Sca1⁺cKit⁺Flt3⁻), CMP (Lin⁻Sca1⁺cKit⁺CD34⁺CD16/32^b), GMP (Lin⁻Sca1⁺cKit⁺CD34⁺CD16/32^{hi}) MEP (Lin⁻Sca1⁺cKit⁺CD34⁺CD16/32⁻) and Gr1⁺ splenocytes were enriched by cell sorting and subjected to real time PCR analysis for 12/15-LOX and normalized to actin gene expression levels (n=4).

plated cells from B6 and Alox15 mice in methylcellulose containing IL-6, IL-3, stem cell factor (SCF) and erythropoietin (Epo) and compared the frequency of colony-forming units-granulocyte (CFU-G) to that of colony-forming units-macrophage (CFU-M). BM cells from Alox15 mice generated an increased ratio of CFU-G to CFU-M compared to BM cells from B6 mice (Figure 39A). BM cells from Alox15 mice gave rise to an increased number of total colonies, due to an increased number of CFU-G and CFU-granulocyte-macrophage (CFU-GM). In contrast, Alox15 BM cells gave rise to a decreased number of CFU-M compared to B6 (Figure 39A). Splenocytes from Alox15 mice had a trend towards increased total number of colonies and also generated an increased ratio of CFU-G to CFU-M. Like BM, splenocytes gave rise to an increased number of CFU-G and CFU-GM (Figure 39B). These data demonstrate that 12/15-LOX indeed regulates myelopoiesis and that absence of 12/15-LOX results in skewing of myelopoiesis towards granulocytic development.

Similar to BM cells plated in methylcellulose in the presence of IL-3, IL-6, Epo and SCF (Figure 40A), BM cells from Alox15 mice grown in the presence of GM-CSF also were skewed towards granulocyte development as indicated by an increased CFU-G/CFU-M ratio compared to B6 (Figure 40A). The skewing towards granulocyte development in the presence of GM-CSF was a result of increased numbers of Alox15 CFU-G colonies and hence enhanced granulopoiesis compared to B6. Similarly, the numbers of CFU-GM were also increased in Alox15 compared to B6 in the presence of GM-CSF (Figure 40A). These data provide additional evidence for enhanced granulopoiesis by

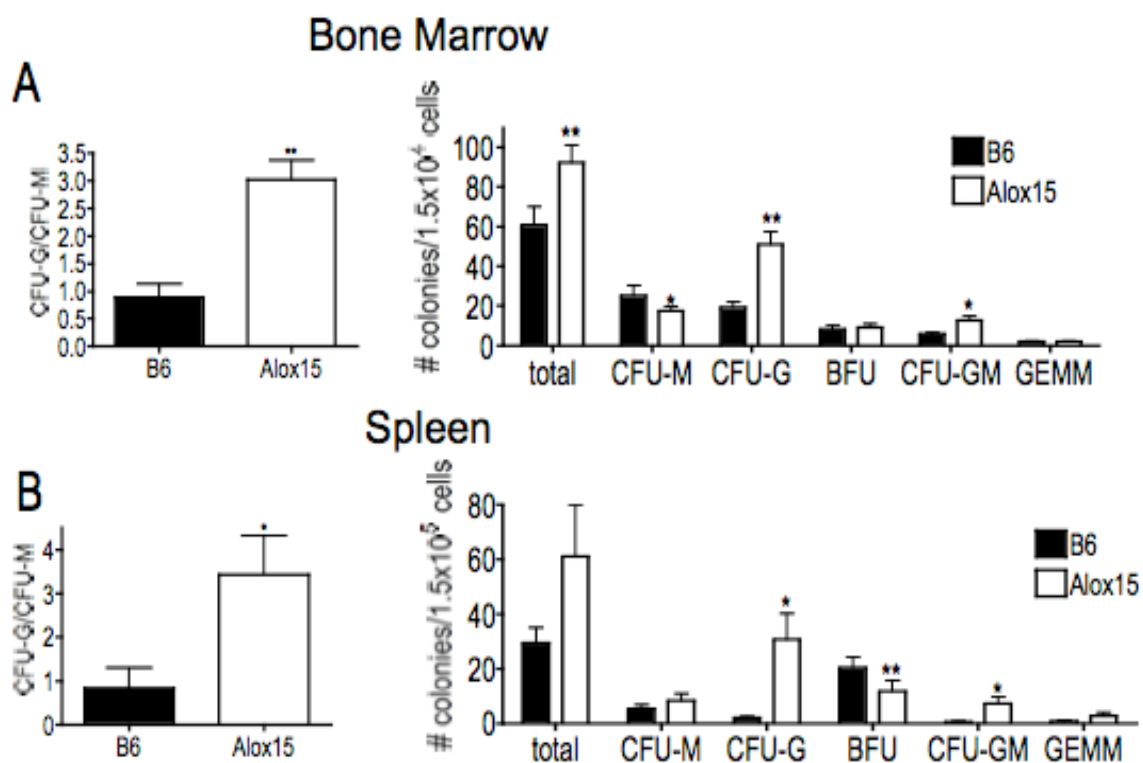
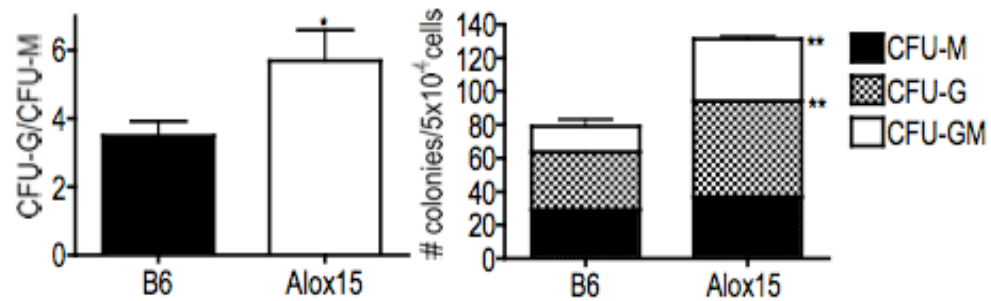


Figure 39. Enhanced granulopoiesis at the expense of monopoiesis of Alox15 progenitors in methylcellulose assays. B6 and Alox15 BM (A) and spleen (B) were plated in methylcellulose assays in the presence of IL-3, IL-6, SCF and Epo. After 10 days, colonies were enumerated using light microscopy. Shown are the ratio of CFU-G/CFU-M and the numbers of colonies ($n=3$).

A



B

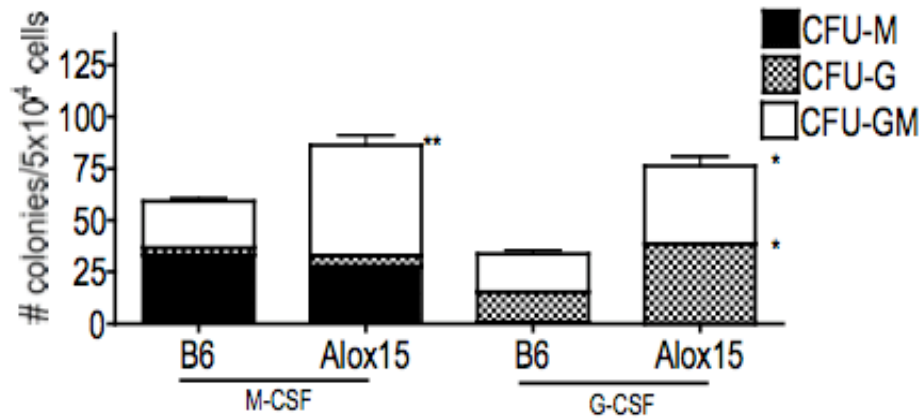


Figure 40. Alox15 progenitors have enhanced granulopoiesis and CFU-GM in the presence of myeloid cytokines. (A) B6 and Alox15 BM were cultured in methylcellulose assays in the presence of myeloid cytokines GM-CSF. Shown are a summary of three experiments as a ratio of CFU-G/CFU-M and phenotypes of CFU-M (black), CFU-G (checkered) and CFU-GM (white). (B) B6 and Alox15 BM were cultured in methylcellulose assays in the presence of myeloid instructive cytokines M-CSF and G-CSF as indicated on the x-axis. Shown are a summary of three experiments and phenotypes of colonies. *p < .05, **p < .01

Alox15 cells.

The cytokines provided in the experiments described above, IL-3, IL-6, SCF and Epo or GM-CSF, do not instruct lineage fate but rather support development of multiple myeloid cell fates. In order to determine the effect of instructive cytokines on the differentiation of B6 and Alox15 BM cells, we plated B6 and Alox15 BM cells in methylcellulose in the presence of M-CSF or G-CSF and quantified colony number and phenotype. Interestingly, cells from Alox15 BM gave rise to an increased number of total colonies in the presence of both M-CSF and G-CSF compared to cells from B6 BM. However, in the presence of M-CSF, cells from Alox15 BM generated more CFU-GM, but not CFU-M. In contrast, in the presence of G-CSF, cells from Alox15 BM gave rise to an increased number of both CFU-GM and CFU-G compared to cells from B6 BM (Figure 40B). Taken together, these data demonstrate that Alox15 BM cells exhibit an increased frequency of CFU-GM and enhanced granulocytic differentiation.

The increased number of total myeloid colonies that developed in methylcellulose assays with Alox15 cells suggested that there might be an increased number of myeloid progenitors in Alox15 BM compared to B6. Specifically, the increase in CFU-GM in the presence of all cytokines including M-CSF, suggest that the GMP population is specifically increased in Alox15 mice.

Accumulation of defective GMP in Alox15 mice

To determine whether 12/15-LOX regulates the numbers of myeloid progenitor populations such as GMP in unmanipulated mice, we employed multicolor flow cytometric analysis to determine the percentage and numbers

of phenotypic progenitor populations. At all ages tested, Alox15 mice had similar percentages of Lin⁻Sca1⁺cKit⁺ cells (LSK, a population enriched for murine HSC), CMP and MEP but an increased percentage of GMP in the BM compared to B6 (Figure 41A-C). Using manual counts of total bone marrow cells coupled with the percentages of the progenitor populations determined by multicolor flow cytometric analysis, we determined that absolute numbers of GMP were also increased in Alox15 BM compared to B6 (Figure 41E). The increased percentage of GMP was also evident in Alox15 spleen (Figure 41D).

There are several potential explanations for the increased number of GMP progenitors in Alox15 mice. First, CMP may produce more GMP at the expense of MEP. Although possible, this is not likely as there were similar numbers of erythroid progenitors (BFU) produced from Alox15 BM in methylcellulose assays (Figure 39A), and similar numbers of MEP in B6 and Alox15 mice (Figure 41). Second, Alox15 GMP may expand by proliferating and/or through enhanced survival. Alternatively, Alox15 GMP differentiation may be blocked resulting in accumulation of GMP as they fail to progress from this differentiative stage.

To determine whether Alox15 GMP exhibit enhanced proliferation, we performed cell cycle analysis of B6 and Alox15 GMP *ex vivo* using incorporation of Hoechst dye, which determines DNA content. B6 and Alox15 GMP had comparable percentages of cells in SG₂M (Figure 42A). Moreover, cell survival was similar between B6 and Alox15 GMP as measured by the percentage of cells expressing AnnexinV, an apoptotic marker, *ex vivo* and after stimulation with SCF *in vitro* (Figure 42B-C). Therefore, Alox15 GMP do not appear to

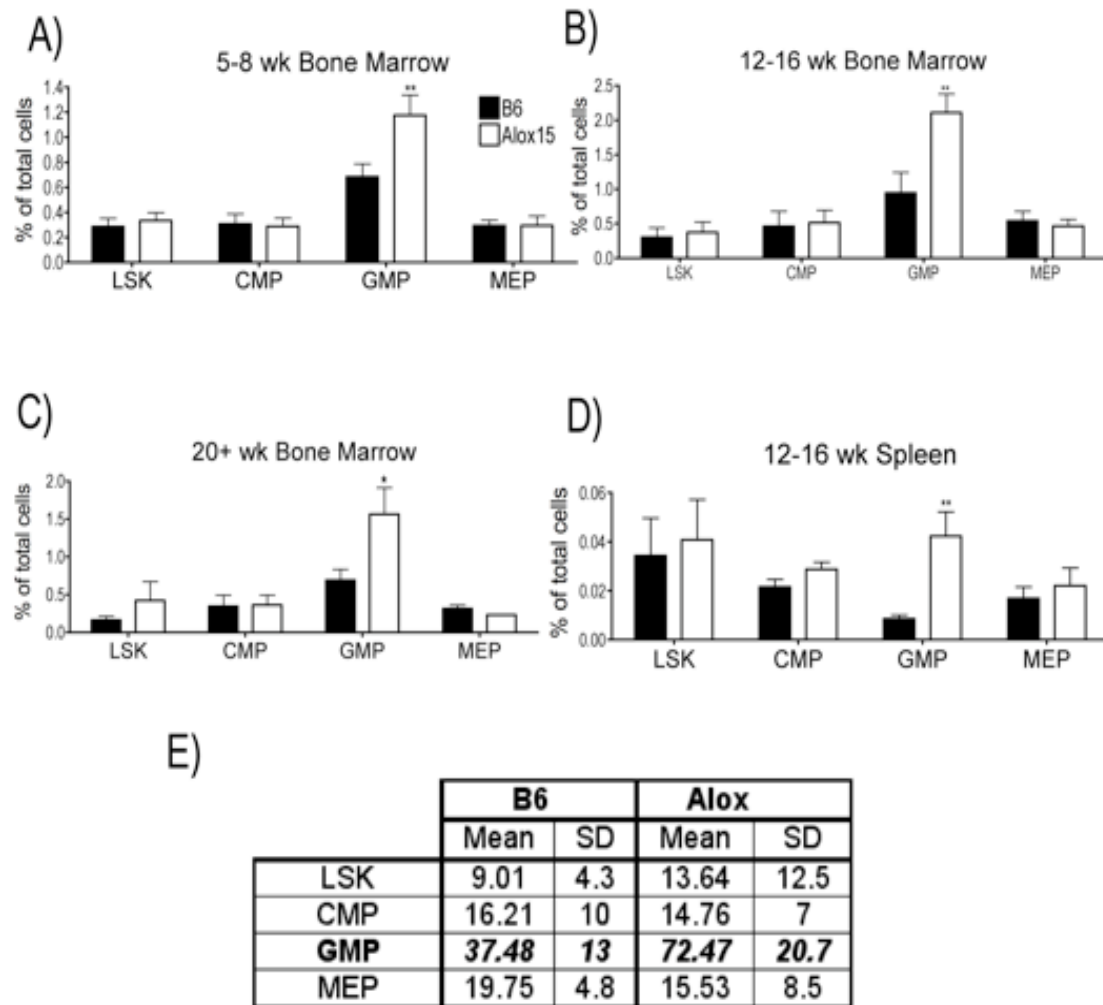


Figure 41. Excess accumulation of GMP in Alox15 mice. BM cells from 5-8 week (A), 12-16 week (B,E), 20+ week (C) and spleens from 12-16 week (D) old B6 and Alox15 mice were isolated and subjected to multicolor flow cytometric analysis to determine the percentage and number $\times 10^4$ (E) of myeloid progenitors: LSK, CMP, GMP, MEP * $p < .05$, ** $p < .01$

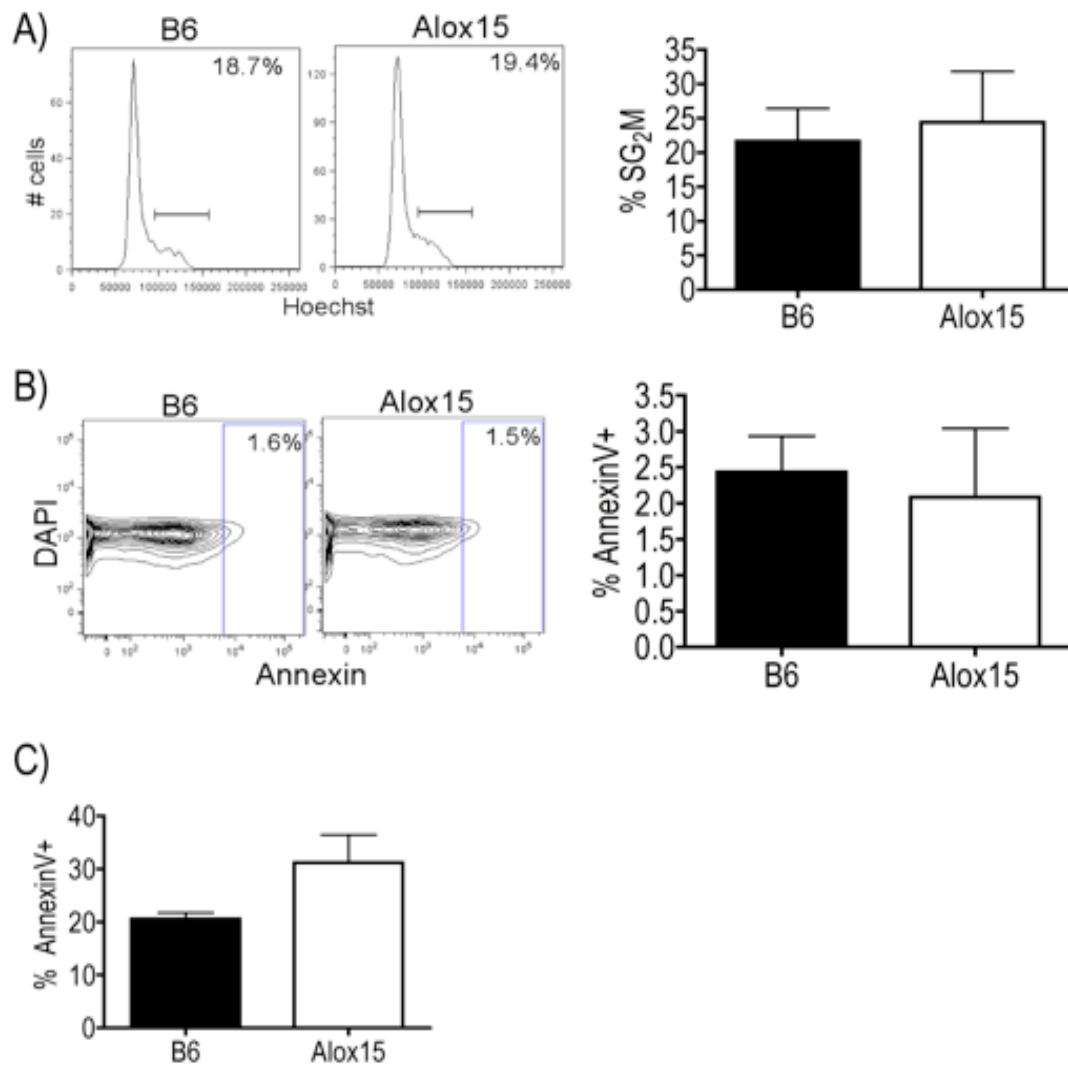


Figure 42. Accumulation of Alox15 GMP is not due to enhanced proliferation and survival.

(A) B6 and Alox15 BM were stained with Hoechst dye to determine cell cycle and subjected to multicolor flow cytometric analysis. Shown are a representative flow cytometric plot gated on GMP and a summary of three experiments. (B) B6 and Alox15 BM was subjected to multicolor flow cytometry and AnnexinV/DAPI staining to determine levels of apoptosis *ex vivo*. Shown are a representative flow cytometric plot gated on GMP and a summary of three experiments. (C) B6 and Alox15 GMP were enriched by cell sorting and cultured in 25 ng/ml SCF *in vitro* for 48 hours. Cells were then stained for AnnexinV/TOPRO to determine apoptosis (n=5).

be increased due to enhanced proliferation or decreased apoptosis.

To test the hypothesis that GMP accumulate in Alox15 mice due to a block in differentiation, we isolated enriched populations of LSK, CMP and GMP from BM of B6 and Alox15 mice by cell sorting and compared their capacity to differentiate in methylcellulose assays. LSK and CMP from Alox15 mice both generated increased numbers of CFU-G but decreased numbers of CFU-M compared to B6 resulting in increased ratios of CFU-G/CFU-M compared to B6 (Figure 43A-B). These data recapitulated results of the methylcellulose assays using whole BM cells.

On the other hand, GMP from Alox15 mice exhibited a defect in the capacity to differentiate in methylcellulose assays. GMP from Alox15 mice generated fewer total colonies, gave rise to significantly fewer M-CFU, and exhibited subtle decreases in CFU-G and CFU-GM. In spite of the subtle decrease in CFU-G, the ratio of CFU-G/CFU-M from Alox15 GMP was increased compared to B6 as we observed for LSK and CMP (Figure 43C). Although these data were consistent with a skewing towards granulocytic differentiation and defective monocytic development, it was not clear whether the defect in Alox15 GMP was specific for monocytic development. To determine the specificity of defective differentiation of Alox15 GMP, we compared differentiation of B6 and Alox15 GMP in the presence of the instructive cytokines M-CSF or G-CSF in methylcellulose assays. GMP from B6 and Alox15 mice produced similar numbers of granulocytic colonies in response to G-CSF demonstrating that Alox15 GMP did not exhibit defective differentiation nor cytokine

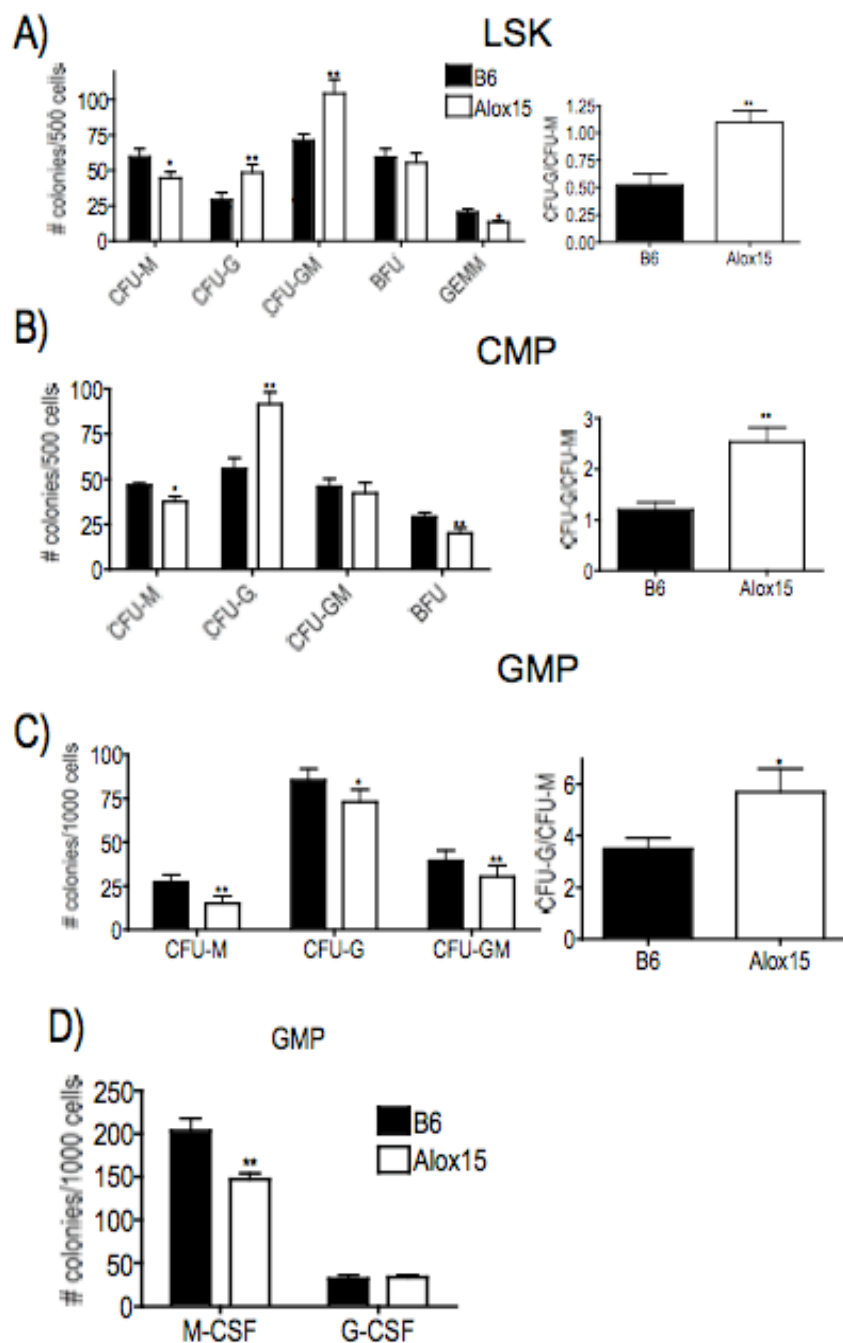


Figure 43. Alox15 GMP exhibit defective monocyte differentiation. LSK (A), CMP (B) and GMP (C) were enriched by cell sorting and plated in methylcellulose in the presence of IL-3, IL-6, SCF and Epo. Shown are a summary of three experiments as colonies enumerated and the CFU-G/CFU-M ratio. (D) B6 and Alox15 GMP were plated in the presence of M-CSF or G-CSF in methylcellulose assays. Shown are a summary of 3 experiments. * $p < .05$, ** $p < .01$

hypersensitivity to G-CSF per se. However, GMP from Alox15 mice gave rise to fewer monocytic colonies in response to M-CSF than GMP from B6 mice (Figure 43D).

These data illustrate that there is an accumulation of GMP in Alox15 mice likely caused by a selective block in monocytic differentiation and decreased exit from this progenitor stage. In the absence of 12/15-LOX, there is enhanced granulocytic development at the expense of monopoiesis. Therefore, I conclude that 12/15-LOX functions to promote monocytic development and inhibit granulocytic development. (Figure 44).

Redox regulation of IRF-8 nuclear accumulation

We previously demonstrated that 12/15-LOX regulated IRF-8/ICSBP nuclear accumulation in mature myeloid cells (Middleton et al., 2006a; Middleton et al., 2006b) and in Lin⁻cKit⁺ immature myeloid progenitors (Chapter 2) (Kinder et al submitted). Moreover, IRF-8/ICSBP is known to critically promote monocyte differentiation and inhibit granulocyte development (Tamura et al., 2000; Tsujimura et al., 2002; Scheller et al., 1999). Therefore, we hypothesized that defective IRF-8/ICSBP transcriptional activity may cause the skewed granulopoiesis and defective monopoiesis in Alox15 mice. To test this hypothesis, we analyzed IRF-8-regulated genes, NF-1 (Zhu et al., 2004) and Egr-1 (Tamura et al., 2000), in CMP and GMP from B6 and Alox15 mice. CMP and GMP from Alox15 mice expressed decreased levels of NF-1 and Egr-1 compared to B6 (Figure 45A) while the mRNA levels of genes important in myeloid differentiation but not regulated by IRF-8, such as PU.1 and c/EPB α (Tamura et al., 2000),

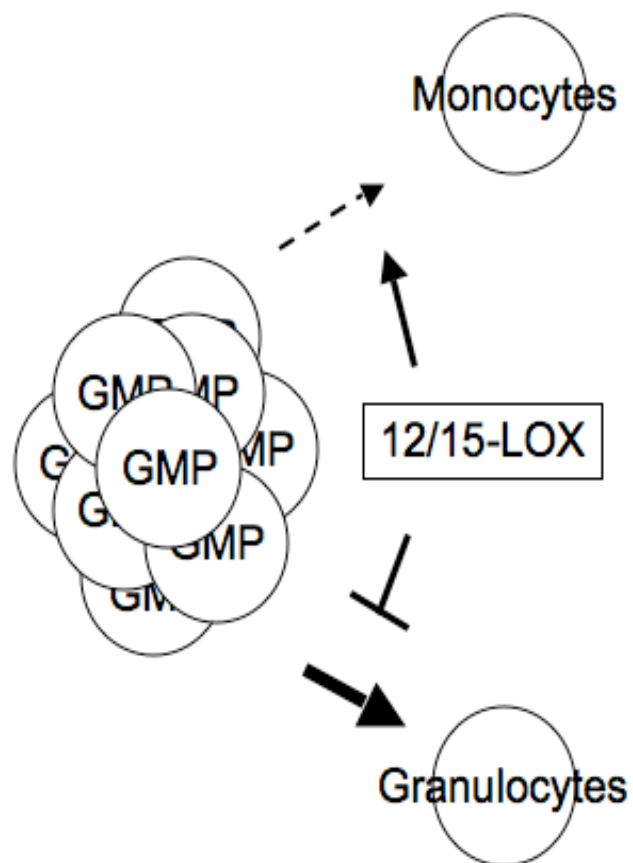


Figure 44. Diagram of GMP differentiation in Alox15 mice. 12/15-LOX inhibits granulocyte development and promotes monocyte development. In the absence of 12/15-LOX, Alox15 GMP accumulate and exhibit defective monocyte differentiation.

were comparable between B6 and Alox15 progenitor subsets (Figure 45A), indicating a selective defect in IRF-8-mediated gene transcription. The defect in IRF-8/ICSBP-mediated gene transcription was not due to decreased expression of IRF-8/ICSBP as levels of IRF-8/ICSBP mRNA were similar between B6 and Alox15 CMP and GMP (Figure 45A). These data are consistent with evidence that 12/15-LOX can regulate IRF-8/ICSCP in a post-transcriptional manner (Chapter 2) (Middleton et al., 2006b).

To determine how 12/15-LOX regulates IRF-8 transcriptional activity in developing monocytes, we used B6 and Alox15 BMM, which recapitulated the defect in NF-1 gene expression and expressed similar levels of IRF-8 in Alox15 CMP and GMP (Figure 45B). To determine which 12/15-LOX-products might regulate IRF-8-mediated gene transcription, we compared levels of lipid metabolites and ROS in B6 and Alox15 BMM. As expected, we found that Alox15 BMM were defective in production of the 12/15-LOX-generated lipid metabolite 12(S)-HETE and in basal levels of ROS compared to B6 (Figure 45C-D). Moreover, Alox15 BMM produced increased levels of the 5-LOX product 5(S)-HETE in response to AA suggesting substrate re-diversion into the 5-LOX pathway (Figure 45C).

To determine the potential impact of the reduction in ROS on IRF-8 mediated gene transcription, we treated B6 BMM overnight with a ROS scavenger, Tiron, which decreased levels of ROS (Figure 46A) and measured transcription of the IRF-8 target gene NF-1. Conversely, we treated Alox15 BMM with BSO, which increases ROS (Figure 46A) by inhibiting synthesis of the

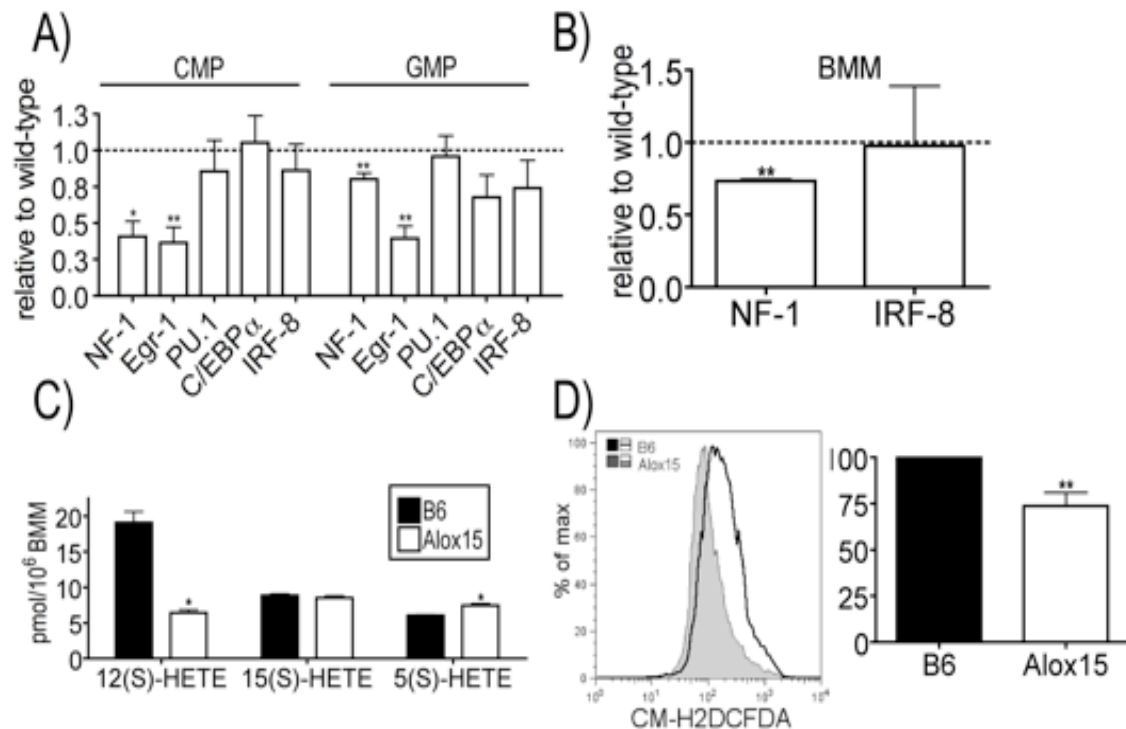


Figure 45. Defective IRF-8 transcriptional activity in Alox15 CMP, GMP and BMM is

associated with altered products of fatty acid metabolism. (A) Real time analysis of enriched B6 and Alox15 CMP and GMP demonstrate that Alox15 myeloid progenitors exhibit decreased levels of the IRF-8-mediated transcripts NF-1 and Egr-1 and similar levels of PU.1, c/EBP α and IRF-8. Gene expression was normalized by GAPDH. (n=3). (B) Alox15 BMM express decreased levels of NF-1 and similar levels of IRF-8 compared to B6 by real time analysis normalized to GAPDH (n=3). (C) Lipid product formation in B6 and Alox15 BMM after stimulation with 50 μ M AA demonstrating decreased 12(S)-HETE and increased 5(S)-HETE in Alox15 BMM compared to B6. (n=4 in two experiments). (D) Flow cytometric analysis of B6 and Alox15 BMM loaded with the ROS sensitive dye H₂DCFDA demonstrate decreased levels of ROS in Alox15 BMM. Shown are a representative experiment and a summary of 5 experiments.

cellular anti-oxidant glutathione. Interestingly, we found that NF-1 gene expression was regulated in part by ROS levels. The ROS scavenger Tiron decreased NF-1 expression in B6 BMM while the ROS inducer, BSO, restored NF-1 transcription in Alox15 BMM (Figure 46B). To determine whether ROS-signaling alters NF-1 transcription by regulating the nuclear accumulation of IRF-8, levels of IRF-8 were quantified in nuclear lysates of B6 BMM treated with Tiron overnight. Both B6 BMM treated with Tiron and Alox15 BMM exhibited a decrease in IRF-8 nuclear accumulation compared to untreated B6 BMM, indicating that low ROS levels contribute to the decreased nuclear accumulation of IRF-8 (Figure 46C). Conversely, elevation of ROS levels in Alox15 BMM by the addition of BSO increased IRF-8 nuclear accumulation (Figure 46D). This regulation was not due to alterations in IRF-8 gene expression, which was similar between treated and untreated B6 and Alox15 BMM (Figure 46E). Moreover, the nuclear levels of ICSBP/IRF-8 with the addition of BSO in Alox15 BMM were restored to the levels observed in untreated B6 BMM, suggesting that redox signaling is the primary mediator of 12/15-LOX-dependent activation of IRF-8 (Figure 46F). These data indicate that 12/15-LOX regulates IRF-8 nuclear accumulation and subsequent gene transcription through ROS-mediated signaling.

Redox regulation of monocyte development in Alox15 progenitors

If 12/15-LOX regulates nuclear IRF-8 accumulation through ROS-mediated signaling and as IRF-8 promotes monocyte development, we expected that increasing ROS-signaling should also restore monocytic differentiation in

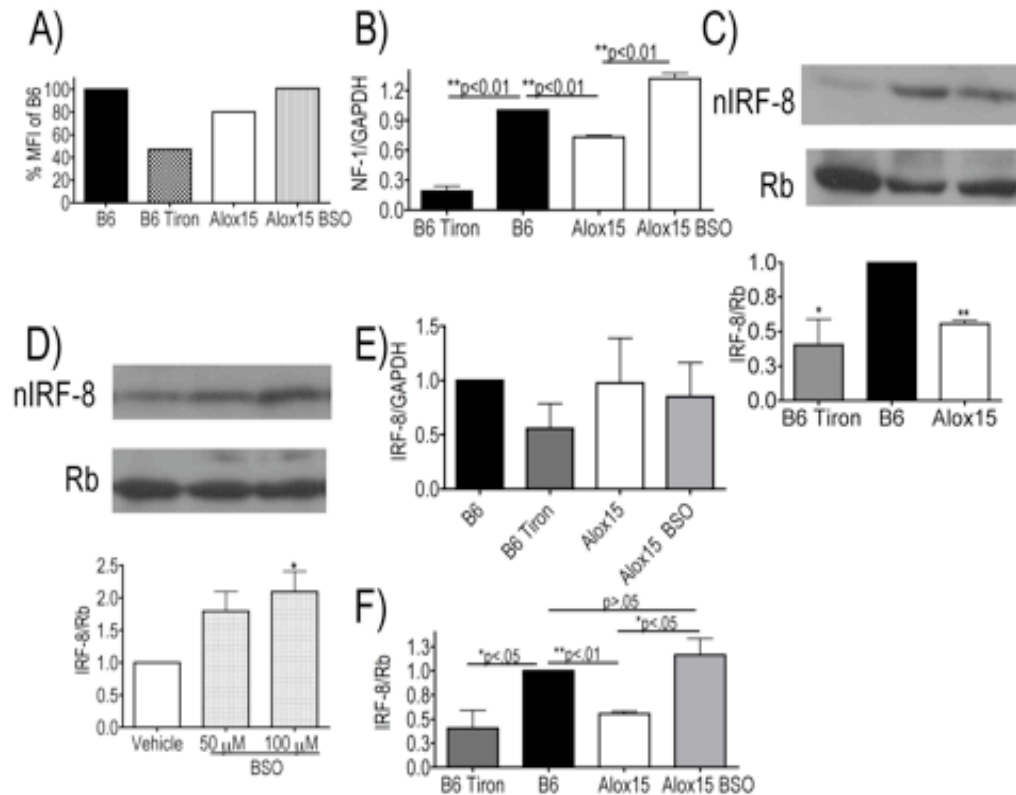


Figure 46. ROS-signaling regulates IRF-8 nuclear accumulation in Alox15 cells. Addition of 10 mM Tiron, a ROS scavenger, to B6 BMM overnight decreases levels of ROS (A) and NF-1 transcription (B) while addition of BSO to Alox15 BMM, increases ROS (A shown at 100 μ M) and NF-1 transcription (B shown at 50 μ M) by real time analysis (n=3). (B) Low levels of ROS decrease IRF-8 nuclear accumulation. B6 BMM in the presence and absence of 10 mM Tiron and Alox15 BMM were cultured overnight demonstrated decreased IRF-8 nuclear accumulation in low levels or ROS. Expression of IRF-8 in nuclear lysates were determined by immunoblot. (C) ROS-signaling increases IRF-8 nuclear accumulation. Alox15 BMM were isolated and cultured alone or in the presence of 50 μ M and 100 μ M BSO overnight. Shown is a representative immunoblot of nuclear lysates and a summary of 3 experiments. (D) IRF-8 mRNA normalized to GAPDH determined by real time analysis demonstrates that 12/15-LOX regulates IRF-8 in a post-transcriptional manner (n=3). (E) Summary of BMM experiments show that BSO restores nuclear IRF-8 accumulation in Alox15 BMM. *p<.05, **p<.01

Alox15 progenitors. To determine the impact of redox signaling on Alox15 myeloid development, we plated BM cells from Alox15 mice in methylcellulose in the presence or absence of BSO. Interestingly, the presence of BSO increased Alox15 monocytic development (CFU-M) and decreased granulocyte development (CFU-G) in methylcellulose resulting in a decreased ratio of CFU-G/CFU-M (Figure 47A). Increasing ROS levels through the addition of BSO also increased monocyte development from Alox15 GMP in methylcellulose assays. GMP from Alox15 mice plated in the presence of BSO generated more CFU-M colonies than in the absence of BSO. This resulted in a decreased ratio of CFU-G/CFU-M in the presence of BSO (Figure 47B). Moreover, addition of BSO to B6 BM cells in methylcellulose had a slight trend towards increased CFU-M, though this was not significant (Figure 48A). The levels of ROS in B6 may be optimal for monocyte development and further increase of redox signaling may have a negative effect on cell survival (Jones, 2008). Taken together, these data indicate that 12/15-LOX regulates monocyte development through redox signaling and restoration of redox signaling in Alox15 progenitors is sufficient to restore monocyte development.

On the other hand, we also tested the effects of 12/15-LOX lipid products on B6 and Alox15 myeloid cell development during methylcellulose assays. 12/15-LOX products had no effect on B6 differentiation. While 12(S)-HETE and 13(S)-HODE had no effect on Alox15 myeloid cell development, addition of 15(S)-HETE to methylcellulose decreased the ratio of CFU-G/CFU-M ratio by Alox15 progenitors (Figure 48B). Others showed that 15(S)-HETE, but not

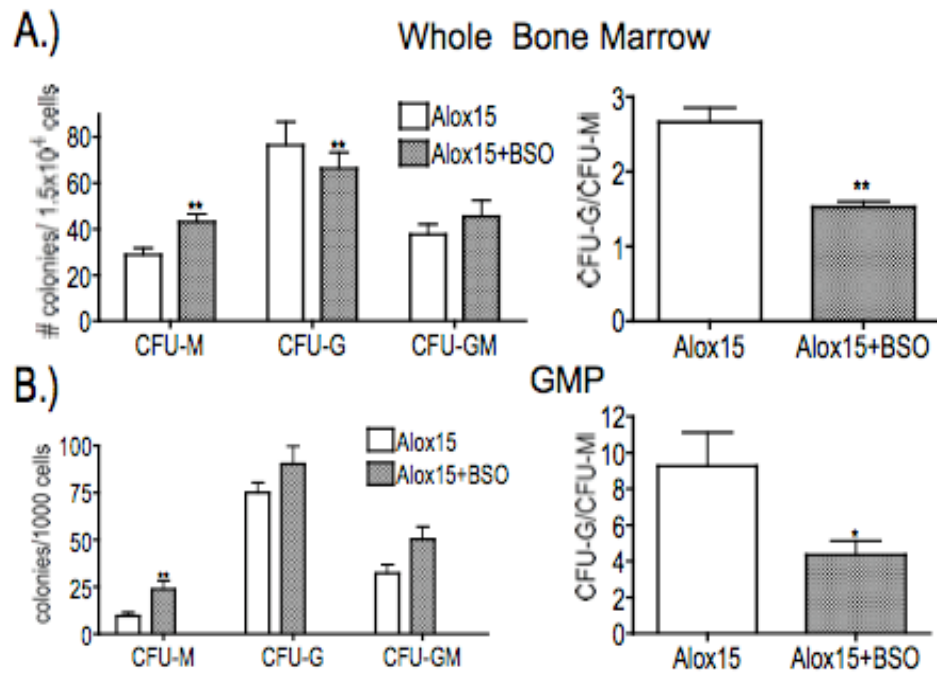


Figure 47. ROS-signaling restores monocyte development in Alox15 BM and GMP.

Alox15 BM (A) and sorted GMP (B) were plated in methylcellulose in the presence and absence of 0.025 μ M BSO. Shown are a summary of three experiments as colony number and ratio CFU-G/CFU-M. * $p < .05$, ** $p < .01$

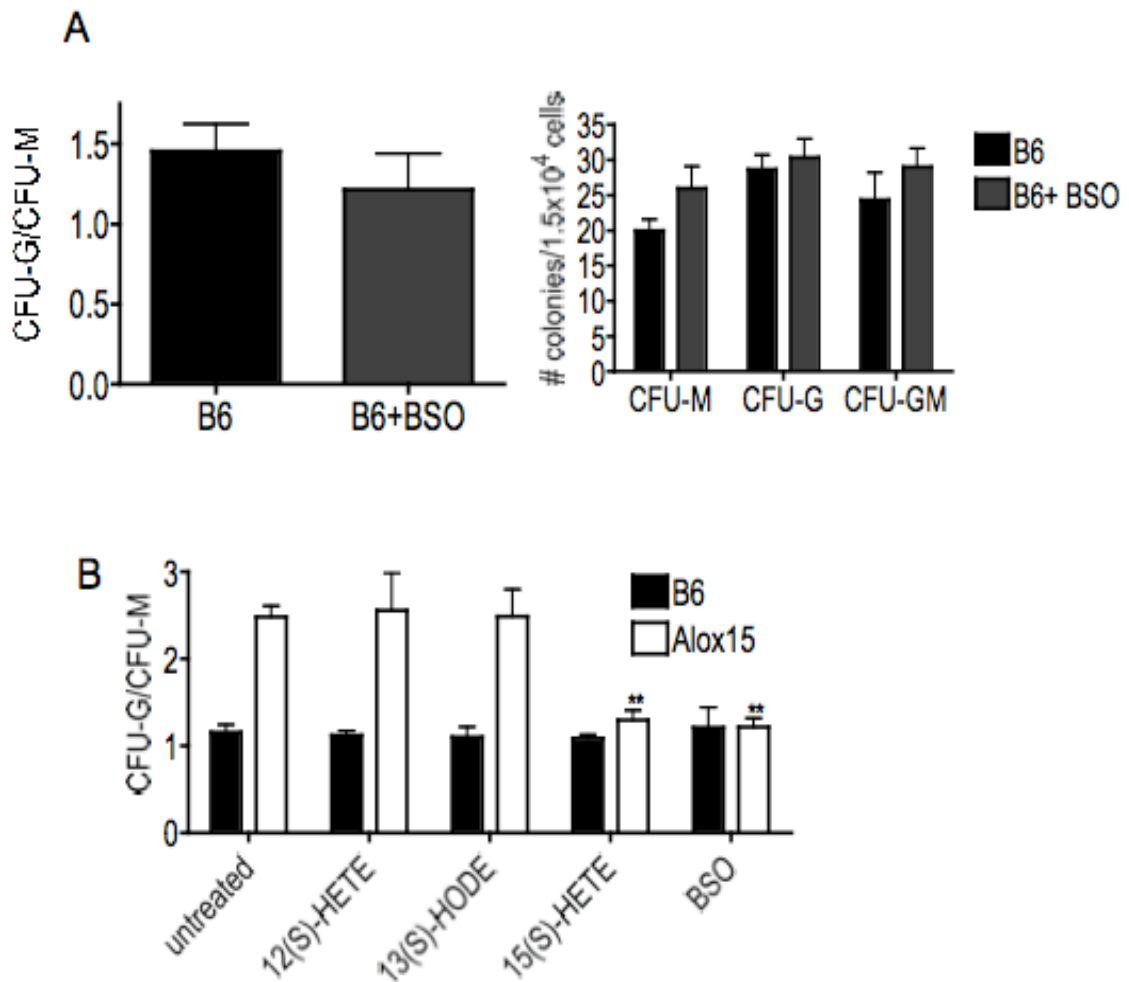


Figure 48. Increasing ROS levels in B6 cells or addition of 12SHETE in both B6 and Alox15 cells have no effect on myeloid cell development. (A) B6 BM was isolated and cultured in the absence or presence of 0.025 μ M BSO. Shown are the numbers of each colony type after 10 days demonstrating that BSO at this concentration had no effect on B6 myeloid development. (B) B6 and Alox15 BM cells were plated in methylcellulose containing IL-3, IL-6 and SCF in the presence or absence of 1 μ M of the indicated 12/15-LOX lipid product or 0.025 μ M BSO. Shown is ratio of CFU-G/CFU-M demonstrating that 15(S)-HETE and BSO selectively regulates Alox15 myeloid development. ** $p < .01$ compared to untreated.

13(S)-HODE, can modulate redox signaling downstream to mediate some of its effects (Mahipal et al., 2007). Therefore, it is possible that 15(S)-HETE regulates myeloid differentiation in Alox15 progenitors through redox signaling. It would be an interesting future direction to discern the exact contribution of 15(S)-HETE signaling to Alox15 myeloid cell differentiation. However, since 15(S)-HETE levels were similar between B6 and Alox15 BMM and Lin⁻ progenitors (Figures 45, 31), I concentrated on the role of 12/15-LOX-dependent ROS signaling which was decreased both in Alox15 BMM and LSK. Moreover, 12(S)-HETE, which is decreased in both Alox15 BMM and Lin⁻ progenitors, had no effect on Alox15 myeloid cell differentiation (Figure 48B).

The data presented thus far demonstrate that ICSBP/IRF-8 is transcriptionally active at relatively high levels of ROS. Therefore at higher levels of ROS a redox modification of cysteine residue(s) in ICSBP/IRF-8 or an upstream signal transducer promotes ICSBP/IRF-8 nuclear accumulation. ICSBP/IRF-8 does contain cysteines in its protein sequence. However, it is likely that modifications of these cysteine residues would inactivate rather than activate its transcriptional activity similar to the action of phosphorylation on ICSBP/IRF-8 transcription of NF-1 (Huang et al., 2006). Moreover, most non-zinc finger transcription factors such as HIF1 α and NF- κ B are regulated by redox signaling by an upstream signal transducer that is the target for redox modifications (Haddad, 2002a). Although I cannot formally exclude that ICSBP/IRF-8 was directly regulated by oxidative modifications, I hypothesized that an upstream regulator of ICSBP/IRF-8 was the target for redox modifications. Indeed,

SHP-1 and SHP-2, which negatively regulate ICSBP/IRF-8 transcriptional activity (Huang et al., 2006; Kautz et al., 2001), are known targets of redox modifications (Chen et al., 2009a; Heneberg and Draber, 2005; Weibrecht et al., 2007).

Therefore, I hypothesized that ROS produced by 12/15-LOX may oxidize SHP-1/2 leading to its functional inactivation. SHP-1/2 activity may be increased at low levels of ROS, such as in Alox15 progenitors and BMM. Moreover, I hypothesized that the higher activity of SHP-1/2 in Alox15 cells may inhibit ICSBP/IRF-8 transcriptional activity leading to a block in monocyte development and enhanced granulocyte development.

To test this hypothesis, we analyzed the effect a SHP1/2 inhibitor, NSC-87877 would have on differentiation of Alox15 BM cells in methylcellulose assays. Increasing concentrations of 10 μ M and 50 μ M NSC-87877 restored monocyte development and decreased granulocyte development in methylcellulose assays with Alox15 BM cells. Inhibition of SHP1/2 with 50 μ M NSC-87877 resulted in an increase in CFU-M and an accompanying decrease in CFU-G (Figure 49A). However, this concentration of NSC-87877 (50 μ M) was recently shown to effect differentiation of wild-type BM cells in methylcellulose assays (Jack et al., 2009). Importantly, we demonstrate that 10 μ M of NSC-87877 had no effect on B6 myeloid differentiation, but still decreased the ratio of CFU-G/CFU-M generated by Alox15 BM cells (Figure 49B). Therefore, dysregulation of SHP1/2 activity may contribute to the skewed granulocyte differentiation of Alox15 progenitors.

Ideally, we would like to compare the activation of SHP-2 in B6 and

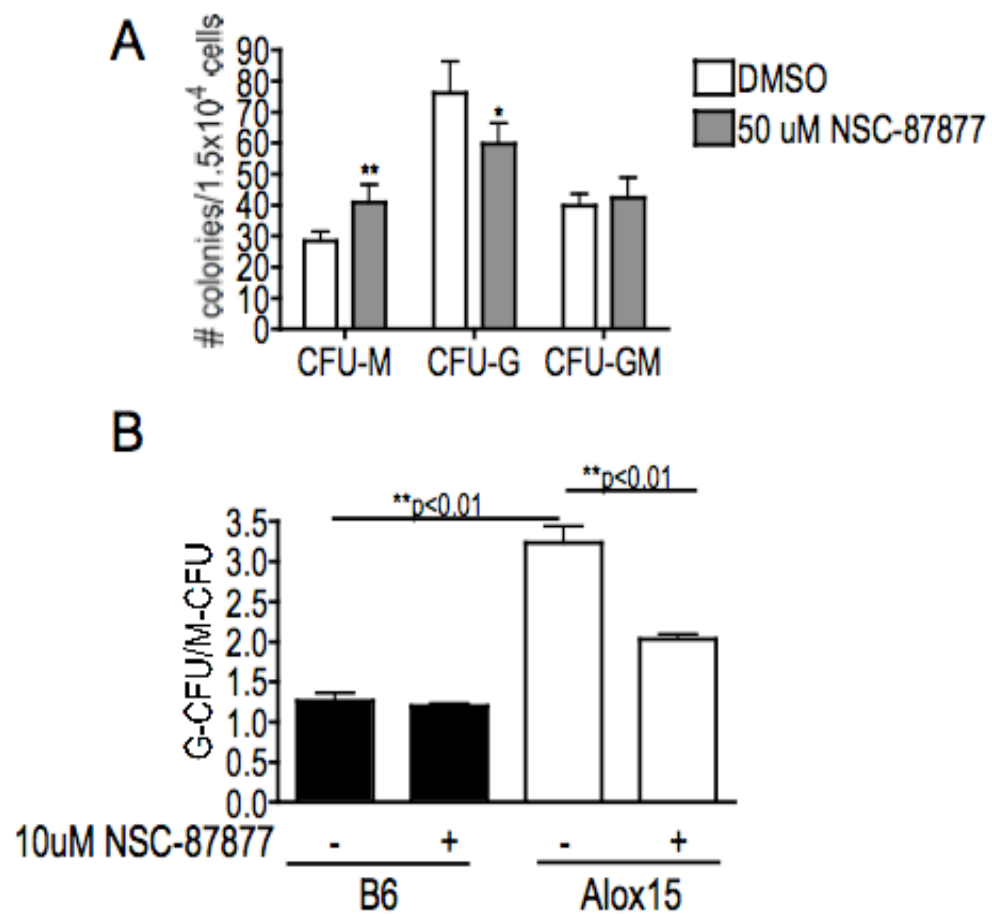


Figure 49. The SHP1/2 inhibitor NSC-87877 restores monocyte development in Alox15.

Alox15 BM was isolated and cultured in methylcellulose assays with IL-3, IL-6 and SCF in the presence or absence of 50 μ M (A) or 10 μ M (B) NSC-87877, a SHP-2 inhibitor. Shown are a summary of three experiments done in triplicate. **p<.01

Alox15 progenitors. Although I have attempted to measure phosphorylated stimulation concentration, progenitor cells and kinetics. Moreover, although SHP-2 in BMM, my conditions were not optimal. I need to further optimize cytokine phosphorylation can increase the activity of SHP-2, it is not required (Lu et al., 2001) and therefore may not be the best measure of its activity. A future direction of these studies would be to measure oxidized SHP-2 in Alox15 progenitors. These studies will also require further optimization, as there is no specific antibody for oxidized SHP-2 and introduction of oxygen to the protein lysates would eliminate differences.

12/15-LOX regulates IL-12p40 expression in a redox-dependent mechanism

The data presented thus far indicate that 12/15-LOX regulates ICSBP/IRF-8 nuclear accumulation through redox signaling in Alox15 progenitors and immature BMM. I hypothesized that this novel pathway may be a common mechanism by which 12/15-LOX regulates ICSBP/IRF-8-dependent gene expression. Our lab previously showed that 12/15-LOX regulates IL-12p40 expression through ICSBP/IRF-8 in mature macrophages (Middleton et al., 2006a). Therefore, we tested whether 12/15-LOX also regulates ICSBP/IRF-8 transcriptional activity of IL-12p40 expression in mature macrophages through redox signaling. We measured levels of IL-12p40 mRNA by real time PCR analysis in thioglycollate-elicited macrophages from Alox15 mice in the presence or absence of 10 μ M BSO following stimulation with 1 μ g/ml LPS and 500 U/ml IFN γ for 4 hours. Addition of BSO restored IL-12p40 expression in Alox15 macrophages (Figure 50A) suggesting that 12/15-LOX regulates IL-12p40

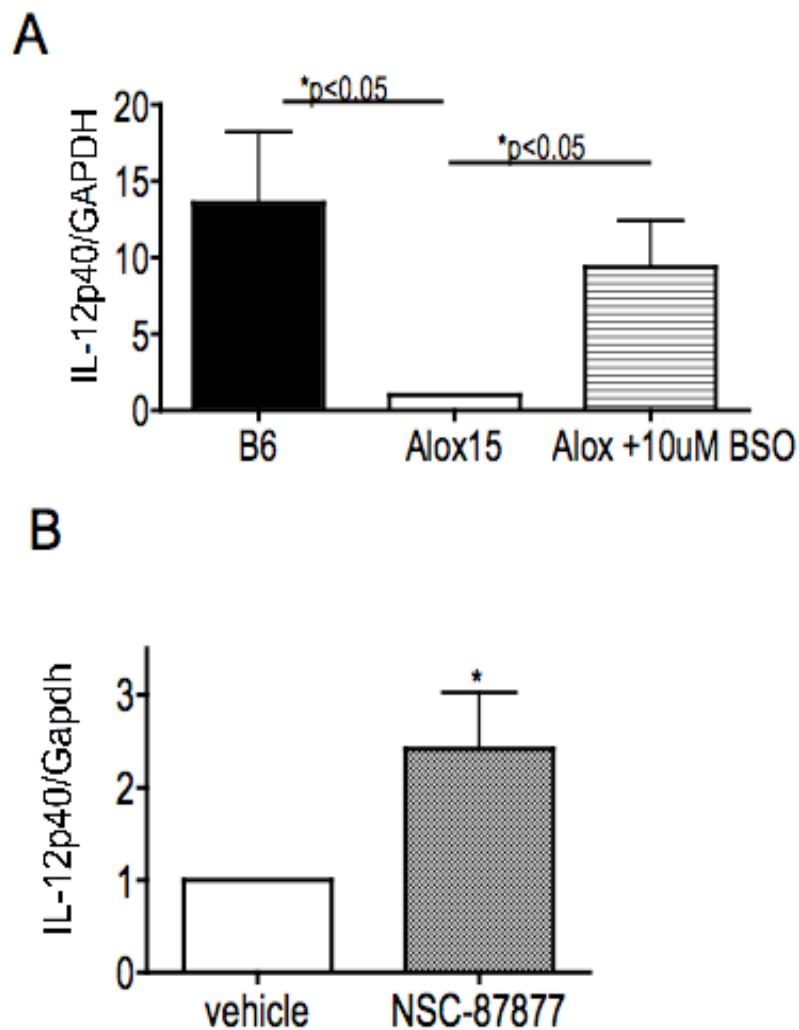


Figure 50. ROS and SHP2 regulate IL-12p40 expression in Alox15 macrophages. B6 and Alox15 macrophages were isolated using thioglycollate. Alox15 macrophages were cultured with 10 μ M BSO (A) or 100 μ M NSC-87877 for 45 minutes prior to stimulation with 1 μ g/ml LPS and 500 U/ml IFN- γ for 4 hours. RNA was then isolated and real time analysis was performed for IL-12p40 and GAPDH. Shown is a summary of 3 experiments. * $p < 0.05$

expression in Alox15 macrophages through redox signaling. These results are consistent with the results in Dr. Middleton's dissertation, in which she treated B6 thioglycollate-elicited macrophages with the ROS scavenger, Tiron, and found decreased levels of ICSBP/IRF-8 nuclear accumulation and IL-12p40 expression. We also tested whether SHP-1/2 regulates IL-12p40 expression in Alox15 macrophages. Addition of 100 μ M NSC-87877 to Alox15 thioglycollate-elicited macrophages also increased levels of IL-12p40 expression (Figure 50B). Therefore, the mechanism by which 12/15-LOX regulates ICSBP/IRF-8 transcriptional activity in immature progenitors is conserved in the regulation of IL-12p40 expression by mature macrophages.

Taken together, these data demonstrate that 12/15-LOX generated ROS likely inhibits SHP1/2 activity. Inhibition of SHP1/2 appears to promote nuclear accumulation of ICSBP/IRF-8 and thus modulates its transcriptional activity resulting in monocyte development and inhibition of granulocyte development in hematopoietic progenitors and IL-12p40 gene expression in macrophages (Figure 51). In the absence of 12/15-LOX, ROS levels are decreased resulting in increased SHP1/2 activity. As others reported, increased SHP1/2 activity leads to de-phosphorylation of ICSBP/IRF-8 that inhibits its association with other transcription factors such as PU.1 (Kautz et al., 2001) (Huang et al., 2006). This dysregulation of ICSBP/IRF-8 results in decreased ICSBP/IRF-8 transcriptional activity leading to enhanced granulocyte differentiation and decreased monocyte differentiation by Alox15 myeloid progenitors and decreased IL-12p40 expression in mature Alox15 macrophages (Figure 51).

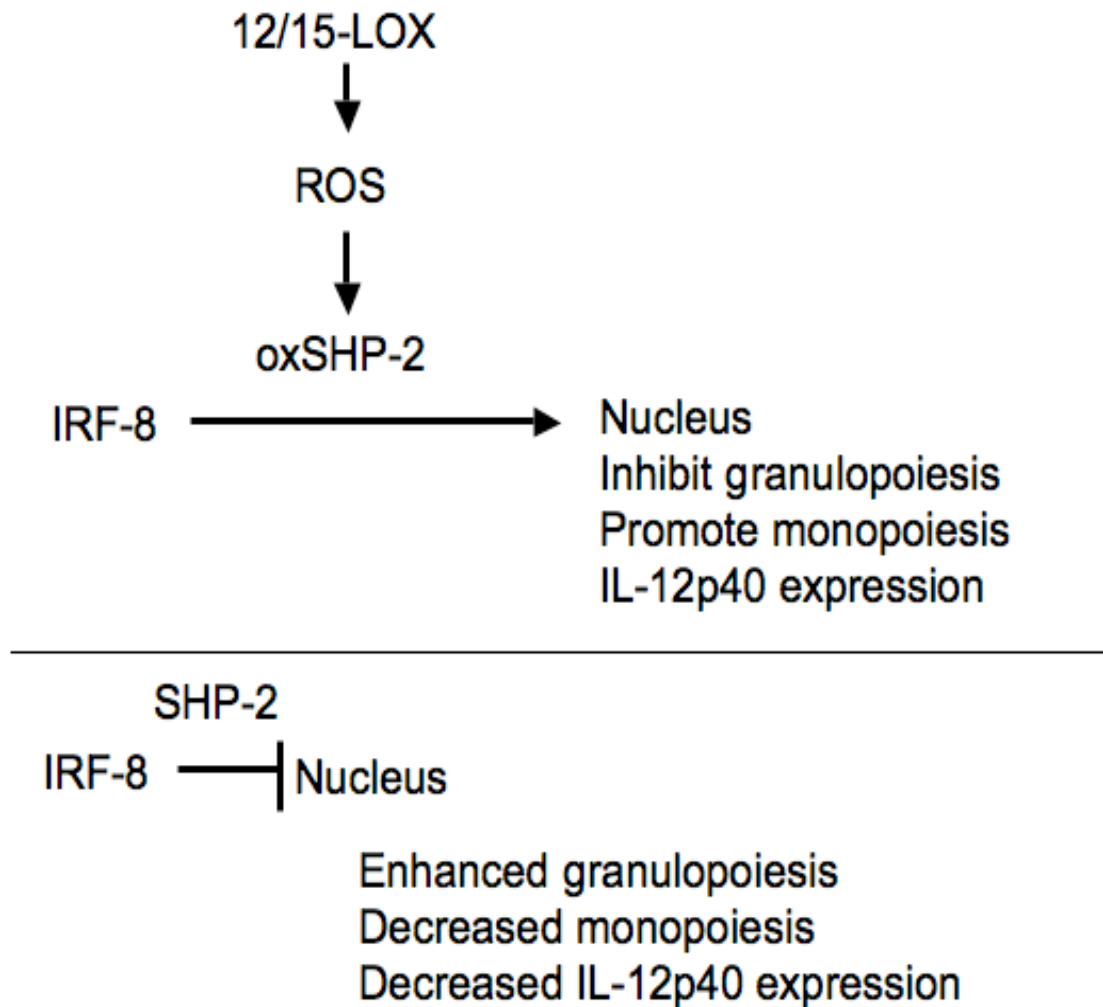


Figure 51. Model of 12/15-LOX regulation of IRF-8 nuclear accumulation. ROS generated by 12/15-LOX oxidatively modifies SHP-2 causing its activation. Inactive SHP-2 allows IRF-8 nuclear accumulation which results in monopoiesis during hematopoietic development and IL-12p40 expression in macrophages. In the absence of 12/15-LOX, SHP-2 activate and inhibits IRF-8 nuclear accumulation leading to granulopoiesis during hematopoietic development and decreased IL-12p40 expression in macrophages.

Implications

In this chapter, I present data that 12/15-LOX promotes monocyte development through redox regulation of the transcription factor ICSBP/IRF-8. Alox15 progenitors exhibit enhanced granulopoiesis at the expense of monopoiesis. The excess accumulation of functional (CFU-GM) and phenotypic GMP in Alox15 mice is accompanied by defective monocyte differentiation, which is likely a result of decreased IRF-8-mediated gene transcription. We showed that 12/15-LOX regulates IRF-8 nuclear accumulation downstream of redox signaling possibly through oxidation of SHP-2. ICSBP/IRF-8 nuclear accumulation and transcriptional activity subsequently promotes monocyte differentiation and inhibits granulocyte differentiation in myeloid progenitors and promotes IL-12p40 expression in mature macrophages (Figure 51).

Because NSC-87877 recognizes both SHP-1 and SHP-2, we cannot discern from these experiments which mediator(s) regulate Alox15 myeloid differentiation. The contribution of SHP-1 versus SHP-2 is also currently not known in the case of normal differentiation of wild-type cells (Jack et al., 2009). Although the role of constitutively active SHP-2 mutations has been well documented in leukemia (Loh et al., 2004; Niihori et al., 2005; Schubbert et al., 2005), the role of SHP-1 during leukemia and myeloid differentiation is less well understood (Bruecher-Encke et al., 2001; Luo et al., 2004). However, both SHP-1 and SHP-2 are regulated by redox modifications of their catalytic cysteine residues (Chen et al., 2009a; Heneberg and Draber, 2005; Weibrecht et al., 2007) and both can bind to ICSBP/IRF-8 (Huang et al., 2006; Kautz et al.,

2001). A future direction would be to determine the impact on SHP-1 and SHP-2 on myeloid cell differentiation in B6 and Alox15 mice in order to further elucidate the pathway by which 12/15-LOX regulates ICSBP/IRF-8 through redox signaling. In addition, an ICSBP/IRF-8 reporter assay could further elucidate the effect of redox signaling on ICSBP/IRF-8 transcriptional activity.

The increased numbers of GMP in Alox15 mice is likely due to an accumulation as a result of defective differentiation. The accumulation of GMP could reflect enhanced proliferation or survival, increased differentiation of CMP to GMP, increased self-renewal or decreased ability of GMP to progress from this differentiative stage. These possibilities are not mutually exclusive. For instance, *gfi-1* regulated the numbers of GMP progenitors through a Hox9A-dependent mechanism that was distinct from the mechanism whereby it regulated terminal granulocyte formation (Horman et al., 2009). In our study, we demonstrated that GMP from B6 and Alox15 mice had similar rates of proliferation and survival (Figure 42) eliminating the first possibility. B6 and Alox15 mice had similar numbers of MEP (Figure 41) and similar numbers of BFU in methylcellulose assays in which BM and LSK were plated (Figures 38, 41). Therefore, it is unlikely that Alox15 CMP are differentiating to GMP at the expense of MEP. The decreased BFU from spleen and CMP may be a result of the defective red blood cell development rather than a decrease in the numbers of MEP as 12/15-LOX is known to regulate red blood cell development (Kuhn and Brash, 1990; van Leyen et al., 1998). Moreover, the absence of 12/15-LOX results in the inability of cells to self-renew (Chapter 2) so it is unlikely that GMP from Alox15 mice were

expanded due to self-renewal. Taken together, our results suggest that the accumulation of GMP is largely due to a block in progression from this progenitor stage as demonstrated by the defective differentiation of Alox15 GMP in methylcellulose assays (Figure 43). However, we cannot formally exclude the possibility that 12/15-LOX also regulates the size of progenitor populations and monocyte differentiation through other distinct mechanisms.

BM cells, splenocytes, LSK and CMP from Alox15 mice gave rise to increased numbers of granulocytes compared to B6. On the other hand, GMP from Alox15 mice did not exhibit enhanced granulocyte differentiation during methylcellulose assays. These data suggest that GMP in the methylcellulose assays are relatively limited in their ability to differentiate to the granulocyte lineage in the presence of IL-3, IL-6 and SCF and a proportion may already be committed to the monocyte lineage. Indeed, GMP represent a stage along the continuum of myeloid cell differentiation and are a heterogeneous population. A proportion of Alox15 GMP may already be committed to become monocytes. However, the defect in monocyte differentiation of Alox15 GMP may result in cell death rather than differentiation to the alternative granulocyte cell fate. Addition of BSO rescues the survival and differentiation of Alox15 GMP committed to the monocyte lineage. On the other hand, BM and spleen contain LSK and CMP, which are more immature and may exhibit a greater capacity to differentiate to the granulocytic lineage resulting in an increased number of Alox15 CFU-G in the methylcellulose assays. Increasing ROS levels by the addition of BSO decreased the number of CFU-G in methylcellulose assays with whole BM. In the

absence of 12/15-LOX, there may be redirection of monocyte lineage into the granulocyte lineage. Indeed, signaling that promotes monocyte development represses granulocyte development including by transcriptional activity of IRF-8 (Friedman, 2007; Tsujimura et al., 2002; Scheller et al., 1999; Tamura et al., 2000). Moreover, when instructed to differentiate to granulocytes by G-CSF, B6 and Alox15 GMP have similar numbers of granulocytic colonies. Therefore, Alox15 GMP are not hypersensitive to G-CSF and skewing of myelopoiesis towards granulocyte differentiation in the presence of non-instructive cytokines occurs as a result of defective monocyte differentiation.

The establishment of a redox-dependent mechanism for ICSBP/IRF-8 nuclear accumulation suggests the possibility that other mediators of redox signaling may also regulate monocyte development. Though it was known that increased levels of ROS promote monocyte to macrophage transition (Yamamoto et al., 2009), our studies implicate an additional role for ROS-signaling at an earlier stage of monocytic differentiation. Our finding that increasing ROS levels by the addition of BSO in Alox15 BM decreased granulocyte development are in agreement with a previous study that demonstrated ROS signaling, generated by dominant negative NF- κ B, inhibited granulocyte differentiation (Nakata et al., 2004). However, the previous study did not examine the effects on mature monocyte development and the ROS generated in the previous study were at levels that mediated apoptosis. Rather, our studies were done at more physiological levels of ROS that did not cause apoptosis. ROS-signaling at physiological levels is known to promote

cytokine receptor signal transduction, including downstream of the cytokines Epo and GM-CSF (Iiyama et al., 2006; Sattler et al., 1999). It is likely that increasing ROS levels in Alox15 progenitors functions similarly to potentiate signal transduction resulting in ICSBP/IRF-8 nuclear accumulation and transcriptional activity and subsequent monocyte differentiation.

The increased levels of 5(S)-HETE in Alox15 BMM may reflect substrate re-diversion into the 5-LOX pathway in the absence of 12/15-LOX (Figure 5C). Interestingly, the 5-LOX pathway has been shown to promote granulocyte development through the production of leukotriene D₄ (Miller et al., 1990). Moreover, inhibition of 5-LOX potentiated monocyte development (Stixova et al., 2009). Although these studies were done on cell lines, they suggest that 5-LOX and 12/15-LOX have opposing roles in myeloid cell fate decisions. A balance of lipid mediator signaling may therefore be important for the regulation of myeloid cell development.

Because leukemia can result from aberrant myeloid cell development, elucidation of the processes that govern myelopoiesis may provide insight into leukemogenesis. Indeed, a small percentage of Alox15 mice developed a myeloid proliferative disease (MPD) over the course of a year, characterized by an accumulation of granulocytes (Middleton et al., 2006b). Moreover, IRF-8-deficient mice, which exhibited a block in monocyte development and enhanced granulocyte development, developed a similar MPD (Holtschke et al., 1996). One can speculate that a block in monocyte development coupled with a skewing towards granulocyte development found in both Alox15 and IRF-8-deficient

mice may cause a favorable scenario for the development of a granulocytic leukemia. Indeed, an accumulation of GMP is also found during human chronic myelogenous leukemia and is believed to be the target of a secondary oncogenic event that drives the blast crisis phase of disease (Jamieson et al., 2004).

Redox regulation of IRF-8 nuclear accumulation and gene transcription may also play an important role during inflammation. Our lab previously showed that 12/15-LOX regulates IL-12p40 transcription in mature macrophages through IRF-8 (Middleton et al., 2006a). In this chapter we demonstrate that 12/15-LOX likely regulates IRF-8 nuclear accumulation and IL-12p40 gene transcription through ROS signaling in peripheral inflammatory macrophages, similar to our findings in immature BMM. Inflammation and inflammatory cytokines can induce ROS production and in turn ROS-signaling contributes to further inflammation and cytokine production (Haddad and Harb, 2005; Lambeth et al., 2008; Shakibaei et al., 2005). Similarly, increased ROS levels during inflammation may inhibit SHP1/2 leading to activation IRF-8 nuclear accumulation and transcriptional activity of inflammatory cytokines in mature macrophages.

In summary, we demonstrate that ROS-signaling regulates IRF-8 nuclear accumulation and subsequent monocyte differentiation in Alox15 mice. This results in an accumulation of defective GMP and a skewing towards granulopoiesis at the expense of monopoiesis in Alox15 mice. Restoration of ROS rescues Alox15 monocyte differentiation. Thus, these data establish a novel role for redox signaling in the regulation of nuclear accumulation and gene transcription by IRF-8 and in monocyte differentiation in Alox15 mice. These

findings have implications for leukemagenesis and inflammation.

DISCUSSION

My dissertation defined novel roles for polyunsaturated fatty acid metabolism mediated by 12/15-LOX during hematopoiesis. I found that 12/15-LOX regulates the development of multiple hematopoietic cells including B cells, T cells, basophils, RBC, and monocytes and the function of HSCs (Figure 52). I demonstrate that disruption of 12/15-LOX signaling in Alox15 mice results in macrocytic anemia, decreased numbers of lymphocytes, impaired HSC function and skewed myeloid cell differentiation. The impaired HSC function and skewed myeloid cell differentiation likely underlie the development of the granulocytic MPN in a portion of Alox15 mice. Moreover, my data suggest that 12/15-LOX may regulate HSC function at least in part through canonical Wnt signaling and implicate redox signaling downstream of 12/15-LOX in the regulation of ICSBP/IRF-8 transcriptional activity and monocyte development. These signaling pathways have implications in the pathogenesis of immunity and cancer. Moreover, my studies are the first to establish a role for polyunsaturated fatty acid metabolism *in vivo* in the regulation of hematopoietic development and HSC function.

Hematopoiesis

Although I have elucidated the mechanisms whereby 12/15-LOX regulates monocyte development and HSC function, the mechanisms whereby 12/15-LOX regulates B cell and T cell development remain of interest especially because 12/15-lipoxygenases have not been previously shown to regulate lymphoid subsets. As we showed that 12/15-LOX regulates ICSBP/IRF-8 in multiple

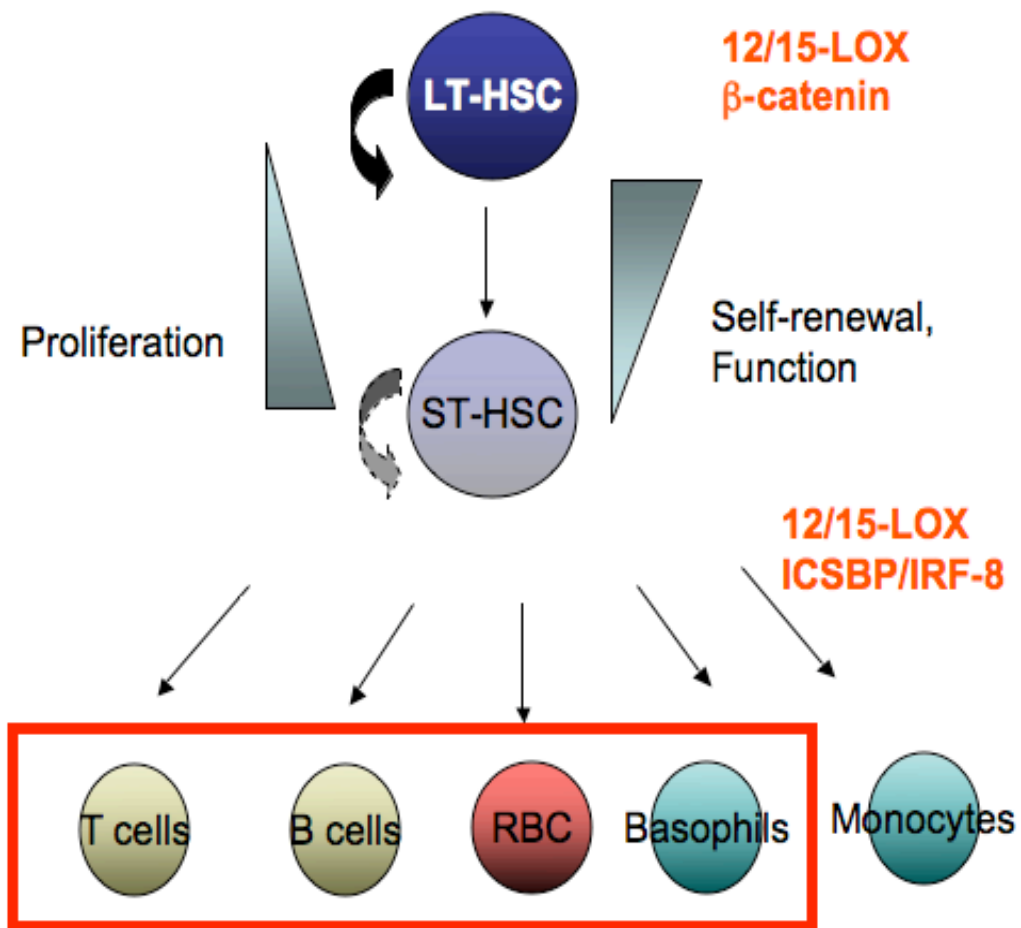


Figure 52. Schematic 12/15-LOX regulates multiple stages of hematopoiesis. I

have demonstrated that 12/15-LOX regulates HSC function through canonical Wnt signaling and β -catenin and monocyte development through the transcription factor ICSBP/IRF-8. 12/15-LOX also regulates development of T cells, B cells, RBC and basophils. However, future studies are required to understand this regulation.

cell types, it is likely that 12/15-LOX also regulates B cell development through ICSBP/IRF-8 as well. ICSBP/IRF-8 and a related transcription factor IRF-4 regulate B cell development at the preB cells stage and are required for rearrangement of the IgG light chain (Lu et al., 2003) in part by mediating transcription of Ikaros and Aiolos (Ma et al., 2008). Moreover, ICSBP/IRF-8-deficient mice and Alox15 mice exhibit similar defects in B cell development (Lu et al., 2003). Although the mechanism whereby 12/15-LOX regulates T cell development is not known, lipoxygenase products are found in the thymus (Harizi et al., 2008) and thus the defect in DP T cells in Alox15 mice may be due directly to alterations in lipid mediators. In addition to lymphoid subsets, the role of 12/15-LOX during DC differentiation could be potentially interesting, although we have not yet explored this possibility. Because subsets of DC and monocytes share a common progenitor (Geissmann et al., 2008) and because ICSBP/IRF-8 is also known to regulate DC cell development (Aliberti et al., 2003; Tsujimura et al., 2003), it is also possible that 12/15-LOX effects DC subsets.

Myelopoiesis

Because the oxidative enzymes involved in unsaturated fatty acid metabolism compete for the same substrates, there may be cross-regulation between cytochrome p450s, cyclooxygenases and lipoxygenases during hematopoietic development due to substrate competition. In the absence of 12/15-LOX, there may be substrate re-diversion and enhanced activity of other fatty acid enzymes. Indeed, we have shown that Alox15 BMM have increased levels of 5(S)-HETE, a product of 5-LOX (Figure 45). 5-LOX has been shown

to regulate granulocyte development through its product Leukotriene D₄ (Miller et al., 1990). Moreover, inhibition of 5-LOX potentiates monocyte development, which is dependent upon p38MAPK activation (Stixova et al., 2009). On the other hand, inhibition of COX-2 has yielded mixed results during myeloid development (Hofmanova et al., 1998) (Bunce et al., 1994). Although, these studies were conducted in cell lines, they demonstrate a seemingly opposing effect for 5-LOX from what we have demonstrated for 12/15-LOX during monocyte development. It is likely a balance of these enzymes and their products regulate myeloid development (Figure 53).

The role of ROS signaling in myeloid development had not been previously established. Although ROS levels were known to increase macrophage development in part by NADPH-oxidase (Barbieri et al., 2003), the role of ROS signaling in immature myeloid differentiation was not previously known. We demonstrated that ROS signaling generated by 12/15-LOX might actually specify monocyte differentiation by promoting ICSBP/IRF-8 nuclear accumulation. Moreover, we implicated SHP-2 as a redox sensitive factor important in the regulation of ICSBP/IRF-8 and in the subsequent specification of cell fate decisions. Although the role of ICSBP/IRF-8 in myeloid cell fate decisions had been well documented (Tsujimura et al., 2002; Tamura et al., 2000), its regulation by redox signaling was not previously known. Because ICSBP/IRF-8 is present in multiple cell types other than myeloid progenitors including B cells, dendritic cells, T cells and macrophages (Nelson et al., 1996; Masumi et al., 2002; Tsujimura et al., 2003; Burchert et al., 2004) the

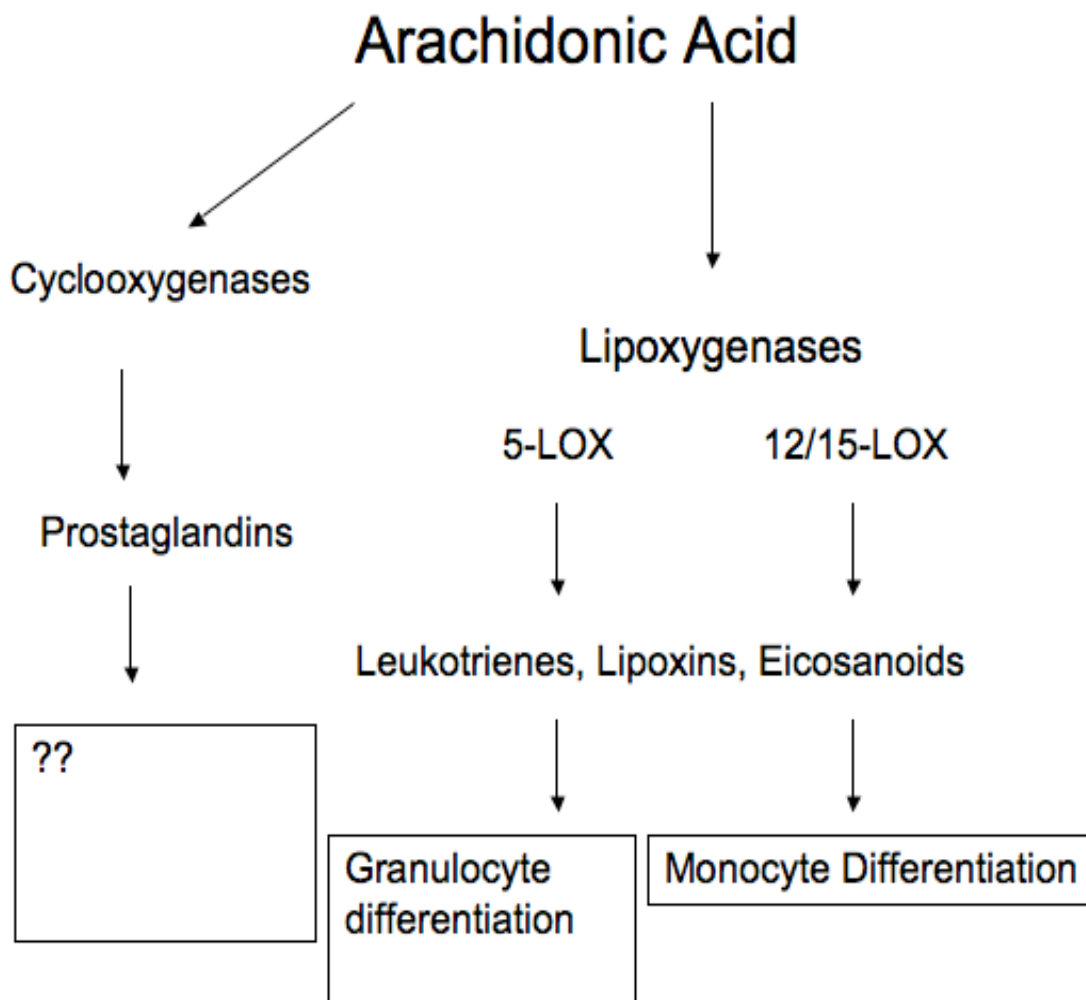


Figure 53. Schematic of fatty acid metabolism in myelopoiesis. Summary of what is known about the role of arachidonic acid mediated fatty acid metabolism in myeloid development. 12/15-LOX and 5-LOX may oppose each other during myelopoiesis. Substrate competition may regulate myeloid cell fat decisions.

regulation of ICSBP/IRF-8 by redox signaling may play a role in multiple cell development and functions. Indeed, we showed that redox signaling might also regulate transcriptional activation of IL-12p40 in macrophages.

There are multiple pathways in which ROS are generated including NADPH-oxidases, mitochondrial function and additional enzymes that participate in fatty acid metabolism (Haddad, 2002a). Therefore, other oxidative enzymes or cellular metabolism may participate in monocyte and granulocyte cell fate decisions through redox-mediated modulation of ICSBP/IRF-8 nuclear accumulation. However, it is possible that 12/15-LOX-mediated ROS production occurs in a site-specific and concentration-dependent manner to specifically regulate SHP-2 and ICSBP/IRF-8 during myelopoiesis and in mature macrophages. Our data from HSCs in Data Chapter 2, support this hypothesis. Although basal levels of ROS were increased in Alox15 LSK, 12/15-LOX-dependent ROS were decreased. Moreover, Alox15 hematopoietic progenitors exhibited decreased nuclear accumulation of ICSBP/IRF-8 and Alox15 LSK had defective levels of ICSBP/IRF-8-mediated transcripts. Therefore, it is more likely that 12/15-LOX-dependent ROS is a specific regulator of ICSBP/IRF-8 transcriptional activity, particularly in LSK. Nonetheless, it would be of interest to determine the selectivity of ROS in the regulation of ICSBP/IRF-8 in various cell types.

Although the role of SHP-2 in myeloid leukemia has been well documented (Loh et al., 2004; Niihori et al., 2005; Schubbert et al., 2005), the regulation of SHP-2 in myeloid cell fate decisions has only recently been

demonstrated (Jack et al., 2009). Moreover, the role of SHP-2 in myeloid differentiation is still under active investigation, as the contribution of SHP-1 versus SHP-2 is not known (Jack et al., 2009). Both SHP-1 and SHP-2 are known to be negatively regulated by oxidative modification (Chen et al., 2009a; Heneberg and Draber, 2005; Meng et al., 2002; Weibrecht et al., 2007) and both can inhibit the transcriptional activity of ICSBP/IRF-8 through its dephosphorylation (Huang et al., 2006; Kautz et al., 2001). Since there was not a selective inhibitor for SHP-1 or SHP-2, we used a phosphatase inhibitor, NSC-87877, which inhibited both SHP-1 and SHP-2. Therefore, either SHP-1, SHP-2 or both may play a role in our signaling pathway by regulating ICSBP/IRF-8 through ROS signaling generated by 12/15-LOX. A future direction would be to delineate the roles of SHP-1 and SHP-2 during monocyte and granulocyte cell fate decisions in both wild-type and Alox15 mice.

HSC function

In addition to myelopoiesis, substrate competition for AA during fatty acid metabolism may also regulate HSC function as summarized in Figure 54. COX-1 regulates formation of the hematopoietic niche while COX-2 directly regulates HSC self-renewal and function (North et al., 2007). Furthermore, the COX product PGE₂ increases the number and function of LT-HSC (North et al., 2007) by increasing homing, survival, proliferation and self-renewal (Hoggatt et al., 2009). PGE₂ functions by stimulating its receptor on HSC, activating PKA and cAMP leading to stabilization of β -catenin by phosphorylation of GSK-3 β and β -catenin (Goessling et al., 2009). Although the specific mechanisms whereby

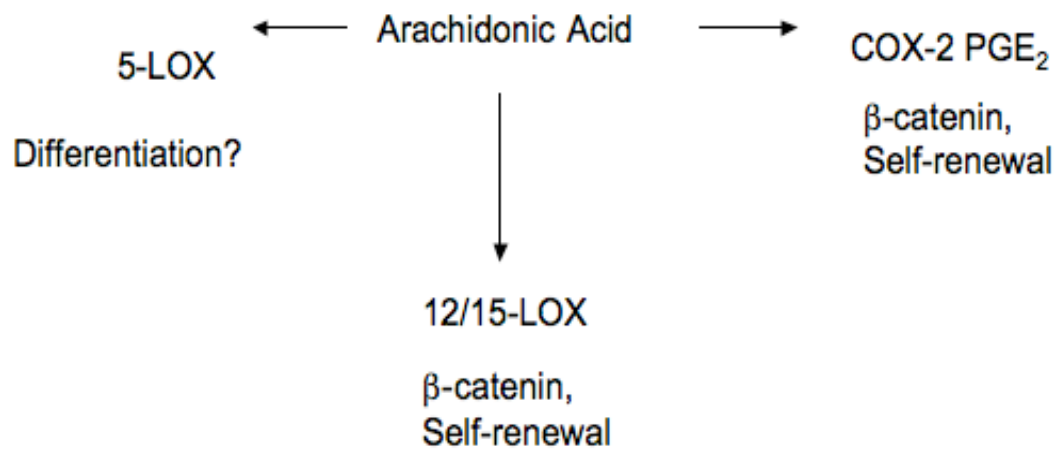


Figure 54. Schematic of Fatty Acid Metabolism in normal HSC function. 12/15-LOX and COX-2 may have similar functions during HSC function, while 5-LOX may play an opposing role.

12/15-LOX and COX-2 regulate canonical Wnt signaling differ, 12/15-LOX ultimately functions similarly to COX-2 by promoting β -catenin stabilization. Interestingly, although 5-LOX does not play a major role in HSC function, it does promote HSC differentiation to some extent (Chen et al., 2009b). Similar to its role during myelopoiesis, 5-LOX signaling may oppose 12/15-LOX signaling, which we demonstrate functions to promote HSC self-renewal.

We demonstrated that basal levels of ROS are increased in Alox15 LSK. The increased basal levels of ROS in Alox15 LSK could be a result of decreased representation of LT-HSC, which express low levels of ROS compared to ST-HSC and MPP and are increased within Alox15 LSK (Jang and Sharkis, 2007). However, the 12/15-LOX-independent ROS in HSC may be generated through NADPH-oxidases (Piccoli et al., 2007), mitochondrial function (Chen et al., 2008) or other fatty acid oxidative enzymes such as 5-LOX (Chen et al., 2009b), which have all been shown to contribute to HSC metabolism. It is possible that the increased basal levels of ROS signal to negatively regulate HSC self-renewal through activation of p38MAPK (Ito et al., 2006). The contribution of ROS by 12/15-LOX-dependent and independent mechanisms require further clarification. Because ROS can be generated by multiple mechanisms, ROS levels differ in various subcellular compartments, such as cell membrane versus mitochondrion, which may play a role in its subsequent signaling. Modulation of ROS levels in a subcellular compartment-dependent manner, will remain a challenge for future studies.

Leukemia

One result of altered hematopoiesis is leukemia. Disruptions in HSC signaling can result in myeloid leukemogenesis (Passegue et al., 2004; Jamieson et al., 2004; Yilmaz et al., 2006). Alternatively, a block in myeloid cell differentiation can also cause myeloid leukemias (Holtschke et al., 1996; Passegue et al., 2001; Mueller et al., 2002). However, these possibilities are not mutually exclusive. Furthermore, we demonstrate that Alox15-deficient mice exhibit disruptions in both HSC function and myeloid cell differentiation. It is likely that both altered HSC function and a block in monocyte differentiation contribute to the development of the CML-like leukemia in a proportion of Alox15 mice (Middleton et al., 2006b).

We demonstrate that 12/15-LOX-generated ROS regulated ICSBP/IRF-8 nuclear accumulation possibly through SHP-2 inactivation. Interestingly, regulation of ICSBP/IRF-8 by SHP-2 had been implicated in leukemia. Constitutively active SHP-2 mutants synergized with haplo-insufficiency of ICSBP/IRF-8 to mediate AML (Konieczna et al., 2008). Moreover, oncogenic constitutively active SHP-2 mutants negatively regulated ICSBP/IRF-8 expression of NF-1 (Huang et al., 2006). Loss of NF-1 expression by inhibition of ICSBP/IRF-8 contributed to leukemia progression (Koenigsmann et al., 2009). Our data proposes a model in which inhibition of 12/15-LOX found in myeloid leukemias (Stenke et al., 1991; Takayama et al., 1983) results in activation of SHP-2 and defective ICSBP/IRF-8 transcriptional activity contributing to leukemia progression.

Because 12/15-LOX regulated ICSBP/IRF-8 through redox regulation

of SHP-2, one would hypothesize that either increasing ROS or inhibiting SHP-2 in Alox15 progenitors during leukemia could restore nuclear ICSBP to treat the CML-like MPN in Alox15 mice. Since Alox15 cells lost transcriptional expression of ICSBP during the leukemic phase of disease (Middleton et al., 2006b), increasing ROS levels or inhibiting SHP-2 in Alox15 mice may be used to prevent the development of leukemia rather than treat active leukemia. On the other hand, increasing ROS levels or inhibiting SHP-2 in Alox15 mice may cause activation of inflammatory gene transcription. Moreover, modulating ROS levels are difficult since their effects are dose-, cell- and subcellular-context dependent.

In the setting of leukemic HSC, fatty acid metabolism mediated by 5-LOX and 12/15-LOX may still have opposing functions (Figure 55). 5-LOX- deficient HSC or treatment of wild-type HSC with a 5-LOX inhibitor protects against Bcr-Abl-induced CML (Chen et al., 2009b) demonstrating that 5-LOX positively regulates leukemic HSC function. During human CML, expression and activity of LTC₄ synthase, an enzyme downstream of 5-LOX, was increased resulting in higher levels of Leukotriene C₄ (Tornhamre et al., 2003). LTC₄ increased proliferation of normal and leukemic hematopoietic progenitors in response to GM-CSF (Snyder et al., 1989; Stenke et al., 1993). On the other hand, we previously demonstrated that 12/15-LOX functions as a tumor suppressor and protects against development of a CML-like disease (Middleton et al., 2006b). Moreover, the common product of 5-LOX and 12/15-LOX Lipoxin A₄ and a 12-LOX product, 12(S)-HETE were decreased during human CML (Stenke et al., 1991; Stenke et al., 1987). Furthermore, 12/15-LOX products promoted

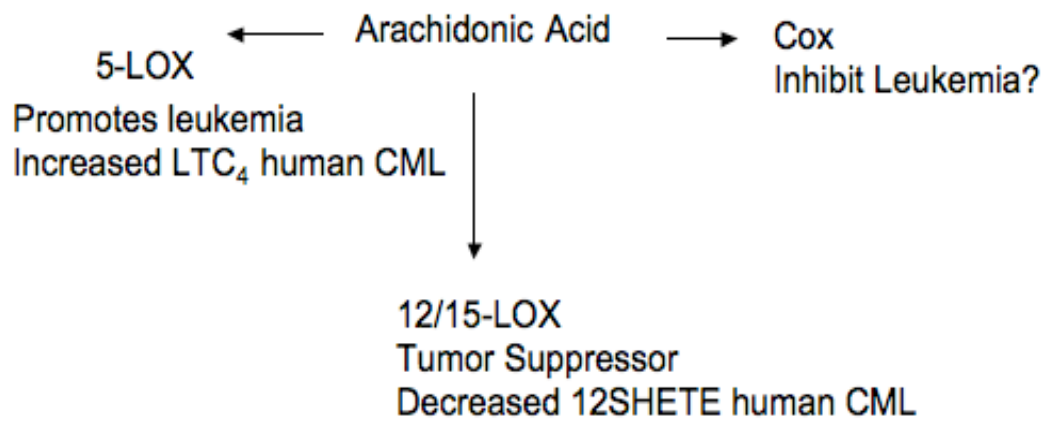


Figure 55. Schematic of fatty acid metabolism in leukemic HSC function. 5-LOX and 12/15-LOX may oppose each other during leukemogenesis. It is possible that substrate competition contributes to the a CML-like MPN in Alox15 mice.

apoptosis of K562 leukemic cell line (Mahipal et al., 2007). Therefore, 5-LOX promotes leukemogenesis and its products are increased during human CML, 12/15-LOX functions as a tumor suppressor and its products are decreased during human CML. It is possible that substrate re-diversion in the absence of 12/15-LOX contributes to the CML-like MPN in Alox15 mice.

While 5-LOX and 12/15-LOX may oppose each other during leukemogenesis, the role of COXs are less understood. While COX inhibitors did not effect proliferation or apoptosis of leukemic cell lines (Snyder et al., 1989), COX products Prostaglandin D₂ and Prostaglandin J₂ induced their cell death (Chen et al., 2005). Thus, the role of COX-1 and COX-2 during leukemic development requires further study.

Immunity

My data demonstrated that ROS signaling downstream of 12/15-LOX mediated IL-12p40 gene expression in macrophages. Although it was previously known that ROS signaling increases inflammatory gene transcription (Haddad, 2002b), we now demonstrate that signaling of ROS specifically generated by 12/15-LOX contributes to inflammatory cytokine gene expression. We identify ICSBP/IRF-8 as a novel target downstream of redox signaling. As we previously showed that NF- κ B-mediated gene transcription of IL-12p40 was regulated by 12/15-LOX (Middleton et al., 2006a) and as it was known that NF- κ B is regulated by redox signaling (Haddad, 2002a), it is likely that ROS signaling mediated by 12/15-LOX also regulates NF- κ B transcriptional activity.

Although we demonstrated that ROS signaling downstream of 12/15-

LOX regulated ICSBP/IRF-8 transcriptional activity, it is also likely that ROS generated independently of 12/15-LOX regulates ICSBP/IRF-8 inflammatory gene transcription. Inflammation and inflammatory cytokines can induce ROS production through multiple mechanisms and in turn ROS-signaling contributes to further inflammation and cytokine production (Haddad and Harb, 2005; Lambeth et al., 2008; Shakibaei et al., 2005). Similarly, increased ROS levels during inflammation may activate IRF-8 nuclear accumulation and transcriptional activity of inflammatory cytokines in mature macrophages.

The role of 12/15-LOX has been extensively studied in the context of chronic inflammation. For instance, it was known that 12/15-LOX contributed to chronic inflammation and immunity in atherosclerosis and toxoplasma infection (Cyrus et al., 2001; George et al., 2001; Zhao et al., 2002; Middleton, et al in press). Moreover, it was known that 12/15-LOX regulated expression of inflammatory cytokines in macrophages (Middleton et al., 2006a; Dioszeghy et al., 2008). However, the signaling pathways whereby 12/15-LOX regulated inflammatory gene transcription were not well understood. My studies demonstrate a mechanism whereby 12/15-LOX may regulate inflammatory gene transcription through redox signaling.

Cancer

In addition to leukemia, fatty acid metabolism mediated by 12/15-LOX may also mediate solid tumor progression. Although my data demonstrate how 12/15-LOX mediates signaling in hematopoietic cell types, it would be interesting to determine whether 12/15-LOX promotes self-renewal during solid tumor

progression similarly to how it regulates self-renewal of LSK. On the other hand, since 12/15-LOX functions as a tumor suppressor in leukemia (Middleton et al., 2006b), it is possible that 12/15-LOX functions as a tumor suppressor during solid tumor progression. Our pathways demonstrate that 12/15-LOX may signal through the immune cell specific transcription factor ICSBP/IRF-8 in hematopoietic cells to mediate their effects. Nonetheless, since non-hematopoietic cells do not express ICSBP/IRF-8, it would be interesting to determine whether there are effects of 12/15-LOX independent of ICSBP/IRF-8 on self-renewal, proliferation and cellular differentiation. Importantly, 12/15-LOX may regulate canonical Wnt signaling and self-renewal independently of ICSBP/IRF-8, possibly in a similar manner as the COX-2 and its product PGE₂ (Goessling et al., 2009).

It was known that fatty acid metabolism of AA contributes to solid tumor progression (Figure 56). While COX-1 is constitutively expressed in the majority of both normal and neoplastic tissues, over-expression of COX-2 in both epithelial and leukocyte compartments has been observed in many cancer types including breast, esophageal, gastric, lung, prostate and pancreatic cancer (Furstenberger et al., 2006). Moreover, use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, which function in part by targeting COX, can reduce incidence and recurrence of several human cancers by up to 50% (Hyde and Missailidis, 2009). More specific COX-2 inhibitors have been used in studies to prevent colon cancer in high-risk populations. However, its long-term use is limited because of increased risk of thrombotic events as described in the

Pro-carcinogenic

COX-2: PGE₂, PFE₂

5-LOX: 5(S)-HETE

P12-LOX: 12(S)-HETE

15-LOX1: 13(S)-HODE

Anti-carcinogenic

15-LOX1 : 15(S)-HETE

15-LOX2: 15(S)-HETE

Figure 56. Fatty acid enzymes and metabolites during cancer progression

play both pro-carcinogenic and anti-carcinogenic functions depending on the lipid mediator involved.

introduction of this dissertation. COX-2 functions to promote cancer by stimulating inflammation, cancer cell proliferation, angiogenesis, cancer cell survival and invasiveness. It signals in part through its lipid products, PGE₂ and PGF₂ to activate RAS-MAPK and Wnt pathways (Furstenberger et al., 2006). Interestingly, COX-2 activates Wnt signaling in multiple cancers similarly to how it activates Wnt signaling in HSC, through inhibition of GSK3 β resulting in increased levels of β -catenin (Lim et al., 2008; Lu et al., 2009; Tuynman et al., 2008). Wnt signaling promotes cell proliferation and self-renewal during cancer, and its constitutive activation is oncogenic.

The role of lipoxygenases during cancer progression is more complicated. The isoenzymes 5-LOX, platelet-12-LOX (p12-LOX) are pro-carcinogenic while 15-LOX-2 (mouse ortholog 8-LOX) is anti-carcinogenic. The role of 15-LOX-1 (mouse ortholog 12/15-LOX) is pro-carcinogenic in prostate cancer but anti-carcinogenic during other cancer types. 5-LOX and p12-LOX are absent from normal tissues, induced by inflammatory stimuli and over-expressed in colon, esophageal, lung, prostate and breast cancer. Meanwhile, 15-LOX-1 and 15-LOX-2 are generally expressed only in benign tissues and not in carcinomas. In some cancers decreased expression of 15-LOX coupled with increased expression of p12-LOX is a prognostic of poor tumor outcome. 5-LOX and p12-LOX increase cancer cell proliferation, survival and invasiveness. Both 5-LOX and p12-LOX promote angiogenesis by stimulating endothelial cell proliferation and inducing VEGF expression. Inhibition of 5-LOX or p12-LOX results in apoptosis of breast cancer, colon, gastric, lung and prostate cancers

(Pidgeon et al., 2007). Moreover, inhibition of multiple LOX and COX in combination (such as inhibition of 5-LOX and COX-2) may be beneficial since substrate re-diversion occurs resulting in increased activation of one pathway when the other is inhibited (Schroeder et al., 2007). On the other hand 15-LOX-2 functions as a tumor suppressor in multiple cancer types by promoting cell differentiation and senescence (Pidgeon et al., 2007; Tang et al., 2007).

Although 15-LOX-1 is anti-carcinogenic for most cancer types, its role in prostate cancer is pro-carcinogenic. 15-LOX-1 expression is correlated to tumor grade in human prostate cancers (Pidgeon et al., 2007) and expression of its murine ortholog 12/15-LOX is induced in multiple mouse models of prostate cancer (Shappell et al., 2003; Kelavkar et al., 2004). In addition, induced expression of 15-LOX-1 in the prostate results in epithelial proliferation and prostatic intraepithelial neoplasia (Kelavkar et al., 2006). The apparent discrepancy in the role of 15-LOX-1 in distinct tumor types can be resolved by comparing the products involved. The lipid mediators 12(S)-HETE and 13(S)-HODE are pro-carcinogenic while 15(S)-HETE is generally anti-carcinogenic (Figure 56). 12(S)-HETE activated NF- κ B, Erk and p38MAPK to stimulate cell growth, angiogenesis and invasiveness. 13(S)-HODE increased MAPK and Akt activation while 15(S)-HETE decreased activation of MAPK and Akt (Pidgeon et al., 2007). Human p12-LOX generates 12(S)-HETE exclusively while 15-LOX-1 generates both 13(S)-HODE and 15(S)-HETE and 15-LOX-2 generates 15(S)-HETE exclusively. 15-LOX-1 may produce different levels of 13(S)-HODE and 15(S)-HETE in different cell types in the prostate. Nonetheless murine 12/15-

LOX produces 12(S)-HETE and 15(S)-HETE at a ratio of 3:1 (Chen et al., 1994) in addition to generating 13(S)-HODE. It is likely that murine 12/15-LOX functions more similarly to human p12LOX because of the higher levels of pro-carcinogenic 12(S)-HETE produced rather than its human ortholog 15-LOX-1 which does not produce a substantial amount of 12(S)-HETE.

The study of 12/15-LOX products during tumor progression stresses the importance of understanding the balance of products and downstream signaling pathways. Substrate re-diversion may also play a role during Alox15 carcinogenesis. It would be interesting to explore whether increased COX and 5-LOX activity could contribute to carcinogenesis in Alox15 mice.

Since human p12-LOX and 12/15-LOX are pro-carcinogenic, it would be of interest to determine whether they promote cancer proliferation through Wnt activation similarly to COX-2. Specifically, because the pro-carcinogenic lipid mediators 12(S)-HETE and 13(S)-HODE were decreased in Alox15 HSC which had defective canonical Wnt signaling, 12(S)-HETE and 13(S)-HODE may regulate Wnt signaling and self-renewal to promote cancer progression. The effect of 12/15-LOX as a tumor suppressor in leukemia may be dependent on ICSBP/IRF-8 since it does not function as a tumor suppressor in solid tumors. Although 15(S)-HETE is anti-carcinogenic in solid tumors, it is unlikely to be anti-carcinogenic during leukemia since its levels are not changed during human CML and Alox15 CML-like MPN (Middleton et al., 2006b) (Stenke et al., 1987).

Summary

In summary, my dissertation demonstrates critical roles for 12/15-LOX

in hematopoietic development of multiple cell types. These are the first studies that effectively demonstrate a role for fatty acid metabolism in hematopoietic development *in vivo*. I have defined novel signaling pathways in HSC function and myeloid development. 12/15-LOX promotes self-renewal and maintains quiescence of HSC through canonical Wnt signaling. Moreover, 12/15-LOX mediates monocyte differentiation and inhibits granulocyte differentiation through redox regulation of ICSBP/IRF-8 transcriptional activity through SHP-2. I have shown that this signaling pathway is also likely intact during macrophage regulation of IL-12p40, which has implications for inflammation and immunity. Moreover, these data provide insight into HSC function and myelopoiesis. The signaling pathways that I have elucidated likely underlie development of the CML-like MPN in Alox15 mice and therefore have implications for leukemogenesis and tumorigenesis.

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